Epidemiology of *Botrytis* spp. associated with neck rot of onion (*Allium cepa* L.) in northern Tasmania, Australia

By

Martin Ian Chilvers, B. Ag. Sci. (Hons.)

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University of Tasmania, Burnie, Australia

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This thesis contains no material which has been accepted for the award of any other higher degree or diploma in any University, and to the best of my knowledge contains no paraphrase of material written or published by any other person except where due reference is made in the text of this thesis.

Martin Ian Chilvers

University of Tasmania

Burnie, Tasmania, Australia

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Abstract

Neck rot of onion, caused by a complex of *Botrytis* spp., is an important fungal disease of onion worldwide. In Tasmania, it has caused considerable losses to the onion industry in some seasons. *Botrytis* infects the onion plant in the field but usually the infection remains symptomless, with the fungus growing into the bulb during curing and producing a rot of the bulb in storage. The taxonomy of *Botrytis* species causing onion neck rot is currently under review. Recent studies of the ribosomal internal transcribed spacer (ITS) region of the genome of *Botrytis* spp. associated with neck rot have confirmed the existence of three distinct groups. A preliminary study of 24 isolates[1] from Australia identified one isolate as *B. aclada* type A1, and 22 isolates as *B. aclada* type AII (*B. allii*) with no isolates of *B. byssoidea* found.

A survey of 16 commercial onion bulb crops in northern Tasmania from October to December of 1999 detected *B. allii* in leaf samples from six crops at an incidence of 0.1% to 0.3%. In three crops that were re-sampled in January 2000, incidence had increased from 0.1% to 3.2%, 0% to 0.5% and 0.3% to 5.8%. Infected crops were found in both main onion-growing regions (northwest and the northeast) in Tasmania. After storage of bulb samples from eight of the surveyed crops, the incidence of *Botrytis* neck rot ranged from 0.4% to 16.3% with an average incidence of 5.3%.

Two field trials were conducted to examine the spatio-temporal spread of *B. allii* during the season from a point source (2000) and line source (2001). Data of *B.*

[1] including one *B. cinerea* isolate for reference

cinerea spatio-temporal spread was also collected. In 2000, 100 plots (1.60 by 4.48 m) were established in a 10-by-10 lattice. Leaf samples were taken six times during the growing season and incubated under high humidity to determine the incidence of *B. allii* in foliage. The incidence of *B. allii* increased from 0.0% at placement of inoculum (150 days after sowing) to a cumulative 3.8% and a non-cumulative 1.8% at 84 days after placement of inoculum (234 days after sowing). The cumulative sample method was based on always treating a plant as infected once it was found to be infected. The non-cumulative sampling method was the incidence found at each sample time. The disease progress curve was described best by exponential and logistic models ($P = 0.001$, $R^2 = 0.99$), indicating secondary spread of the pathogen. The disease gradient was steep and best described by an exponential model, usually indicative of a rain splash-dispersed pathogen. Spatial analysis using ordinary runs analysis, beta-binomial distribution analysis, spatial analysis by distance indices and radial correlation analysis determined that the pattern of infection was aggregated.

In 2001, two plots of 100 quadrats were established in 10-by-10 lattices, the two plots were subject to different irrigation schedules late in the crop life. Leaf samples were collected on five occasions. The source of inoculum was an onion bed sown with *B. allii* infected seed (28.9%), which ran through the middle of both plots. However, only one of 530 plants sampled between 85 and 132 days after sowing from this bed was infected with *B. allii*. Therefore, the inoculum bed was sprayed with *B. allii* spores to ensure a *B. allii* epiphytotic. *Botrytis allii*-infection in plots adjacent to the inoculum bed increased from 0.0% incidence in both irrigation treatments to 4.1% (non-cumulative) after 43 days of exposure to inoculum (181 days after sowing) in the high irrigation treatment and 1.7% in the low irrigation treatment. The incidence of

Botrytis bulb rots in the high and low irrigation treatments was 6.1% and 3.1% respectively. The *B. allii* disease progress curve in the high irrigation treatment fit linear, monomolecular and Gompertz models well, with backtransformed R^2 values of 0.88, 0.88 and 0.69 respectively. The disease progress curves in the low irrigation treatment did not significantly fit any of the backtransformed models. As in the first field trial, the disease gradients were steep and best described by exponential models. Spatial analysis using ordinary runs analysis, beta-binomial distribution analysis and spatial analysis by distance indices determined that the pattern of infection was aggregated.

Several controlled experiments were performed to examine spore release and dispersal. Spore trapping was conducted with a Quest volumetric spore-trap and filter traps located close to a source of inoculum. *Botrytis allii* spores were also collected in rainwater at distances from a source of *B. allii* inoculum, suggesting that spores are spread with rain droplets as they splash. The spore dispersal gradient observed in the rainwater traps was steep. The steep spore gradient was supported by the steep disease gradients of *B. allii* observed in the field, suggesting that precipitation events are important in dispersal of this fungus. However, *B. allii* can be released into the air in a dry state, as demonstrated by the detection of spores on trap plants, filter traps and the Quest volumetric spore-trap in the absence of rainfall events. Dry spore dispersal over longer distances may also play an important role in the epidemiology of *B. allii*.

To examine the ability of *B. allii* to survive in soil, sclerotia were placed in nylon bags and buried in a field. Bags were recovered at intervals and sclerotia placed on water

agar and examined for germination. A proportion (13%) of the sclerotia were viable after 2.5 years.

A technique was investigated for predicting the incidence of rot in storage from the incidence of *B. allii* in tissue samples taken from bulbs at harvest and incubated for 7 days under high humidity. For 32 field crops assessed over two years, the correlation coefficient between predicted and actual bulb rot was not significant. However, when four outliers were removed from the data set the correlation coefficient was $r = 0.77$ ($P = 0.01$). A linear regression of the data set was significant ($P < 0.001$) with an $R^2 = 0.58$ and an exponential regression analysis had an $R^2 = 0.68$ ($P < 0.001$). Under controlled field conditions the accuracy of prediction was improved with a significant linear regression ($P < 0.001$) with $R^2 = 0.94$. However, further development would be required for this technique to be used as a commercial test.

The effect of three benzimidazole fungicides on a *B. allii* epiphytotic was examined in a randomised block design field trial, artificially inoculated with a spore suspension of *B. allii*. In plots not treated with fungicide, the incidence of *B. allii* in leaves increased from 0.0% to 23.8% 7 days after application of inoculum, to a maximum of 33.8% at 49 days after application of inoculum. *Botrytis. allii* was not detected in the leaves from plots treated with higher application rates of the fungicides tested. All fungicide treatments resulted in significantly ($P < 0.05$) less bulb rot than the bulbs from untreated plots. The mean incidence of bulb rot in the untreated, least effective and most effective fungicide treatments was 63.4%, 9.9% and 1.0% respectively.

This thesis provides new and valuable information on the epidemiology of *B. allii* in Tasmania, providing a basis for crop management protocols and future research to understand, manipulate and control this commercially significant disease.

Chapter 1

1 Literature review

1.1 Onions

1.1.1 Botany

Onions (*Allium cepa*) have been grown since early times for their pungent flavour and medicinal properties. The precise origin of the onion cannot be located with certainty but it is considered to be a native of central or western Asia (Fenwick and Hanley, 1985). Onions have been cultivated by a number of civilisations over the ages. The ancient Egyptians used it as a food and for embalming ceremonies, while the ancient Greeks and Romans also cultivated the onion and gave account of its medicinal properties. Indian scripts dated back to the 6th Century BC gave account of the use of onions in medicines. The genus *Allium* is comprised of 600 species worldwide (Fenwick and Hanley, 1985), and includes the well known onion, garlic, leek, shallots, chives and spring onions.

1.1.2 Onion markets, Tasmanian industry - Onion production

The Tasmanian onion industry is mainly focused on the export market. Most onion production occurs along the north west coast of Tasmania, with some production in the north east. Tasmania produces less than a third of the national crop, but accounts for over 80% of Australia's export onions. Onions are exported to various locations, with emphasis on the United Kingdom, continental Europe and Asia. The cost of transport impacts heavily on returns, as shipping costs account for at least 40% of the gross return per tonne of onions shipped (Department of primary industries water and

environment, 1999). The majority of Tasmanian onion production is concentrated around the cream-gold varieties occasionally referred to as the brown onion. Some red and sweet onions are also grown.

The Tasmanian onion production for the 1996/97 season was 59,677 t from 1192 ha at an average yield of 50.1 t/ha and a gross value of $16.3 million (DPIWE, 1999). For the 2001 season it was estimated that there would be 42,700 t and 4000 t of red onions, with no tonnage of white onions reported (Australian onion industry association, 2001).

1.1.3 Onion cultivation in Tasmania

Onion seed is sown with air seeders into a fine seedbed. Between 7 and 9 rows (12.5-16 cm between rows) are sown into a 1.0 m-wide bed with 1.6 m between wheel centres of field equipment. Relatively high levels of nitrogen (N), phosphorus (P) and potassium (K) are required as onions have thick, shallow roots (Fenwick and Hanley, 1985). The amounts recommended by the Tasmanian onion industry are 500 kg/ha of 6.14.14 N.P.K banded at sowing, and a side dressing during crop growth of 30 kg/ha of N, applied once or twice depending upon crop vigour. Good irrigation practice is also essential to maximise yield. Tasmanian onions are sown from May through to September, and are harvested from January through March.

Fungicides are applied on a calender basis for the prevention of onion downy mildew (*Peronospora destructor*). Insect pests are also sprayed for as required. Thrips are commonly the worst insect pest in onions in Tasmania. Herbicides are also an essential tool for crop management, as onions do not form a closed canopy later in the

season like most other crops, resulting in greater competition from weeds germinating and growing between plants compared to other crops.

1.1.4 Onion harvesting and processing

The first step in harvesting onions is to lift the bulbs mechanically, usually with a twin row lifter. The lifter has a bar that runs just below the bulbs to sever the roots. The bulbs are then carried up and over a chain belt to remove soil from the bulbs. Once the bulbs have been lifted they are left in windrows to field-cure. A second pass is made with lifting equipment to turn the bulbs and promote drying. The drying can take as little as two weeks given ideal (dry) curing conditions. Some onion companies have also practiced field topping during the field-curing phase. Removing the tops allows the windrows of onions to dry quicker and results in easier transport of a less bulky crop. When sufficiently field-cured, the bulbs are harvested with a harvester towed behind a tractor and transported to factories. The onion bulbs are further cured in storage facilities or immediately processed. Bulbs have their tops and roots mechanically removed with a top and tail machine and are mechanically graded into various sizes and hand graded into the different classes for various markets, with the removal of visibly diseased or damaged bulbs. Bulbs are packed into the appropriate onion sacks or direct into containers required for shipping.

1.1.5 Seed production

Seed for the Tasmanian onion industry is produced in isolation in the Derwent Valley, Hobart to try and minimise the risk of seed crops becoming infected by potential seed-

borne pathogens from commercial crops. Seed is also imported from seed companies based on mainland Australia or from New Zealand.

The majority of seed that is produced in Tasmania is open pollinated, where mass selection is used to improve lines. Bulbs of ideal character (mother bulbs) are taken from crops, planted and give rise to inflorescences, which are open pollinated. As seed is produced by open pollination, there is a large amount of genetic variation present. Companies are moving towards hybrid seed production, so that the onion plants are genetically more similar than in open pollinated cultivars. This gives rise to a more uniform-sized onion bulb crop. However, obtaining breeding material for producing hybrid seed is more difficult than the mass selection method.

1.2 Onion neck rot

1.2.1 Impact of neck rot on the Tasmanian onion industry

During the 1995/96 season there was an unusually wet period of weather post-lifting from January to March. This led to high levels of onion neck rot and poor quality of bulbs, which reduced exports for that season by about a third. The financial losses which ensued resulted in amalgamation of some onion companies (Dennis, 1996a).

The main onion packer in Tasmania is currently Field Fresh Tasmania, formed after the amalgamation of Clements and Marshall, Roberts and Webster Horticulture. Field Fresh is responsible for marketing approximately 70% of Tasmania's onions. The other major onion companies are Harvest Moon and Perfecta Produce.

1.2.2 Symptoms

Onion neck rot is caused by species of *Botrytis*. Symptoms of disease are difficult to detect in the paddock as the fungus infects plants as a quiescent infection without producing symptoms. Only senescent material under favourable conditions will develop symptoms and signs of *Botrytis* infection. Identification in the field is near impossible, as the ubiquitous *B. cinerea* appears very similar to the *Botrytis* species causing neck rot. Once bulbs have been harvested and placed into storage, infected bulbs will develop neck rot symptoms (Figure 1.1). Infected onion bulbs develop a semi-watery decay, which usually begins in the neck. This decay eventually moves down through the entire bulb. Grey or white mycelia develop between scales as well as short grey conidiophores. Sclerotia may form on the outer scales around the neck or sometimes around the base (Figure 1.1).

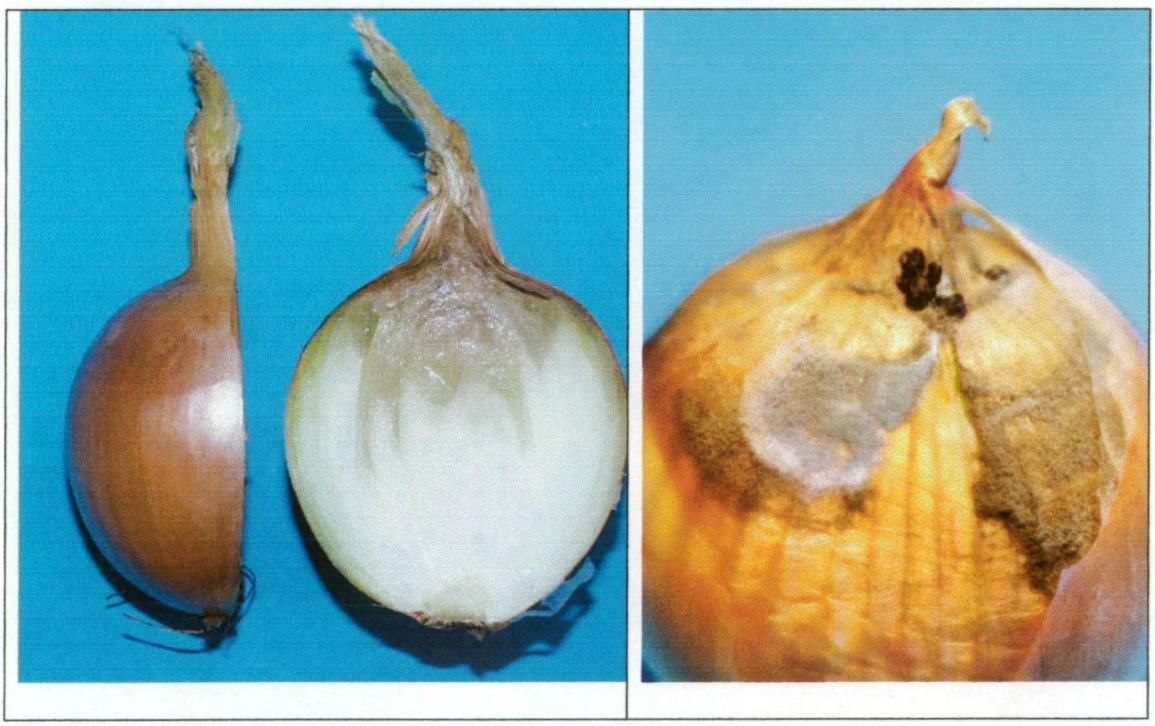

Figure 1.1: Onion neck rot symptoms and signs caused by *Botrytis allii*.

1.3 Botrytis genus

1.3.1 *Botrytis* genus – general information, taxonomy, etc.

Botrytis is Greek for "bunch of grapes", describing the appearance of the conidiophore bearing conidia. The genus was erected by Micheli in 1729 and was one of the first described fungal genera. The genus included a number of related and unrelated taxa, growing to about 380 taxa before being redefined by Whetzel (1945) (in Coley-Smith *et al..,* 1980) and Hennebert (1973). Hennebert (1973) describes 22 species belonging to the genus *Botrytis.* All of the known teleomorphs are in the genus *Botryotinia* Whetzel. A good overview of *Botrytis* taxonomy is provided in Jarvis (1980).

Three predominant *Botrytis* species have been shown to cause neck rot of onions: *B. aclada* type I, *B. allii* (syn. *B. aclada* type II) and *B. byssoidea.* A total of seven *Botrytis* species have been reported to cause disease of onions. *Botrytis squamosa,* which can cause neck rot on white cultivars exclusively, is the causal agent of onion leaf blight (Lacy and Lorbeer, 1995a). In the past there has been much confusion regarding these three species as differentiation between *Botrytis* species can be difficult morphologically. However, Nielsen *et al..* (2001) have resolved much of this confusion with the use of molecular techniques.

1.3.2 *Botrytis aclada, B. allii* and *B. byssoidea*

In 1850, Frensenius named the fungus *B. aclada.* In 1917, Munn named *B. allii* as causing neck rot of onions in New York. Roed (1952) and Hennebert (1963) suggested the subdivision of *B. aclada* into small-spored and large-spored types. In

1973, Hennebert examined herbarium material and concluded that *B. allii* (Munn) was the same as *B. aclada* – suggesting that *B. aclada* should have been the name used for this pathogen. However, *B. allii* has remained the name in use in some countries, particularly in the United States.

Of the *Botrytis* species capable of causing onion neck rot, *B. allii/B. aclada* is the most prevalent. However, *B. byssoidea* has been isolated more frequently in some years in some locations. Walker (1925) reported that mycelial neck rot caused by *B. byssoidea* was more destructive than that caused by *B. allii* in the Wisconsin and Illinois regions of the United States. *Botrytis byssoidea* is differentiated in culture by the production of white fluffy mycelium and sparse production of conidia in culture. *Botrytis byssoidea* has a teleomorph (*Botryotinia allii* (Sawada) Yamamato): however, there are no records for *B. allii* or *B. aclada* having a sexual stage.

Debate has occurred as to whether *B. aclada* and *B. byssoidea* are two distinct species. This confusion has possibly been due, in part, to the two spore size types of *B. aclada* and the fact that the spore sizes of *B. aclada* and *B. byssoidea* overlap (Hennebert, 1963; Presly, 1985). Owen *et al.* (1950) and Morgan (1971) concluded *B. aclada* and *B. byssoidea* to be two separate species. Lacy and Lorbeer (1995b) reported *B. aclada* (type I and II undistinguished) to be conspecific with *B. byssoidea.*

Shirane *et al.* (1989) demonstrated that *B. allii* isolates could be divided into two groups on the basis of spore size, one group having a mean length and diameter of 10-11 μm x 5-6 μm (volume, 170-200 μm^3), and the other 8-9 μm x 4-5μm (volume 88-104 μm^3). Spores of *B. byssoidea* were different from *B. allii,* with mean length and

diameter being 23 μm x 15 μm (volume 2,805 μm^3). The average number of nuclei per conidia in the *B. allii* isolates was 1.3-1.5, whereas the average number of nuclei per conidia in the *B. byssoidea* isolate was 5.0-5.1. The data collected by Shirane *et al.* (1989) suggested that *B. allii* and *B. byssoidea* are not conspecific. The isolates of *B. allii* could also be divided into two groups based on the number of chromosomes they contained, with the smaller spores containing 16 chromosomes whereas the larger spore types contained 32. All of the other *Botrytis* spp. tested contained 16 chromosomes, including *B. byssoidea, B. cinerea, B. squamosa* and *B. tulipae.* Shirane *et al.* (1989) suggested that the 32 chromosomal *B. aclada* group was an auto-diploid of the 16 chromosomal *B. aclada* subgroup.

With the use of molecular techniques, Nielsen and Yohalem (2001) demonstrated that the *B. aclada* AII was genetically distinct from *B. aclada* AI and that the former most likely originated as a polyploid hybrid of *B. aclada* AI and *B. byssoidea* or a very close relative. Studies by Nielsen *et al.* (2001) have shown that mis-identification of some isolates has occurred as a result of using morphological techniques alone.

1.3.3 *Botrytis squamosa* (teleomorph *Botryotinia squamosa* Viennot-Bourgin)

B. squamosa J.C. Walker is the causal agent of onion leaf blight. The leaf blight lesion first has a white, necrotic centre about 2 mm in diameter surrounded by a light green halo about 1-1.5 mm in width (Schwartz and Mohan, 1995). The halo is characteristic in the early stages of lesion development. With age, the lesions expand slightly and take on an elliptical shape. Under prolonged moist conditions at temperatures between 12-24°C, the fungus develops rapidly and causes blight of the leaves (Schwartz and Mohan, 1995).

Onion leaf blight can be devastating given the right environmental conditions and lack of appropriate control measures. Although *B. allii* was reported to cause onion leaf blast (Segall and Newhall, 1960), Hancock and Lorbeer (1963) demonstrated that it does not cause foliar damage beyond minor leaf spot.

1.3.4 *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*)

Botrytis cinerea is a general saprophyte and a pathogen, which can cause a number of postharvest disorders in a number of fruits and vegetables. In onions, *B. cinerea* causes 'coffee staining', sometimes referred to as brown stain, on the outer dry scales. The markings are only superficial (Figure 1.2) but can affect the value of the crop. Some of the outer scales affected with brown stain can slough off during harvest and packing, leaving unmarked scales below.

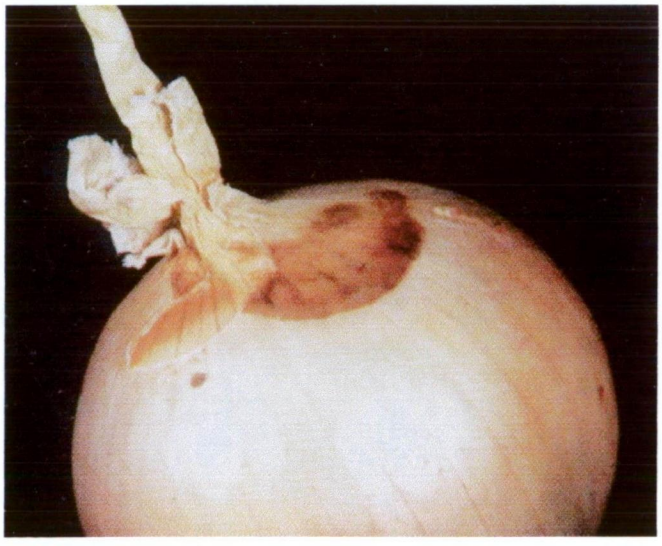

Figure 1.2: Staining of onion skin caused by *B. cinerea* often termed by the onion industry as coffee staining (image courtesy of Field Fresh Tasmania)

1.4 Epidemiology of *Botrytis allii/aclada*

1.4.1 Host range

Botrytis allii has been reported to infect common onion (*Allium cepa*), garlic (*A. sativum*), leek (*A. porrum*), shallot (*A. cepa* var. *ascalonicum*), and potato or multiplier onions (*A. cepa* var. *aggregatum*) (Schwartz and Mohan, 1995). No alternative hosts for *B. allii* outside the *Allium* genus have been reported. Wild hosts of the genus include *Allium vineale* and *A. ursinum* (Maude and Presly, 1977b). *Allium vineale* has been reported to be present in Tasmania. There are three species of *Allium* that have been introduced to Tasmania and are now weed species: *A. vineale* (crow garlic) *A. triquetrum* (three-cornered garlic) and *A. neapolitanum. Allium triquetum* is recorded as a widespread roadside weed in the north west, midlands, north east and east coast (Curtis and Morris, 1994). *Allium* weeds may therefore act as potential sources of inoculum for commercial crops.

Tichelaar (1967) observed *B. allii* to be living saprophytically on decaying plant materials (cereals, lucerne, bean and pea). However, this is the only report of saprophytic survival on hosts other than onion and has not been confirmed since. It is possible that other *Botrytis* species were misidentified as *B. allii* in the study by Tichelaar (1967).

1.4.2 Life cycle of *Botrytis allii*

A simplified life cycle of *B. allii* is presented (Figure 1.3), emphasising survival and inoculum sources of *B. allii.* Spores produced on infected volunteers and cull piles may also infect healthy crops. Possible alternative hosts are not included. *Botrytis allii*

can enter crops through infected seed (Maude and Presly, 1977a; Stewart and Franicevic, 1994). *Botrytis allii* infections on senescent plant material can produce conidia under favourable conditions, which can infect plants under favourable conditions (Kritzman *et al.*, 1981). *Botrytis allii* infections on onion leaves progress down the leaves and into the necks of the bulbs as the leaves senesce, which results in neck rot and may result in the production of sclerotia on the bulbs. *Botrytis allii* sclerotia can reside in the soil for up to six months. If favourable conditions are present (wet or moist), sclerotia germinate, produce conidiophores and release conidia (Maude *et al.*, 1982). Infected mother bulbs selected for seed production can introduce *B. allii* into a seed crop and result in infection of the flowers, from where *B. allii* infection of the seed results (Kritzman, 1983a) (Figure 1.3).

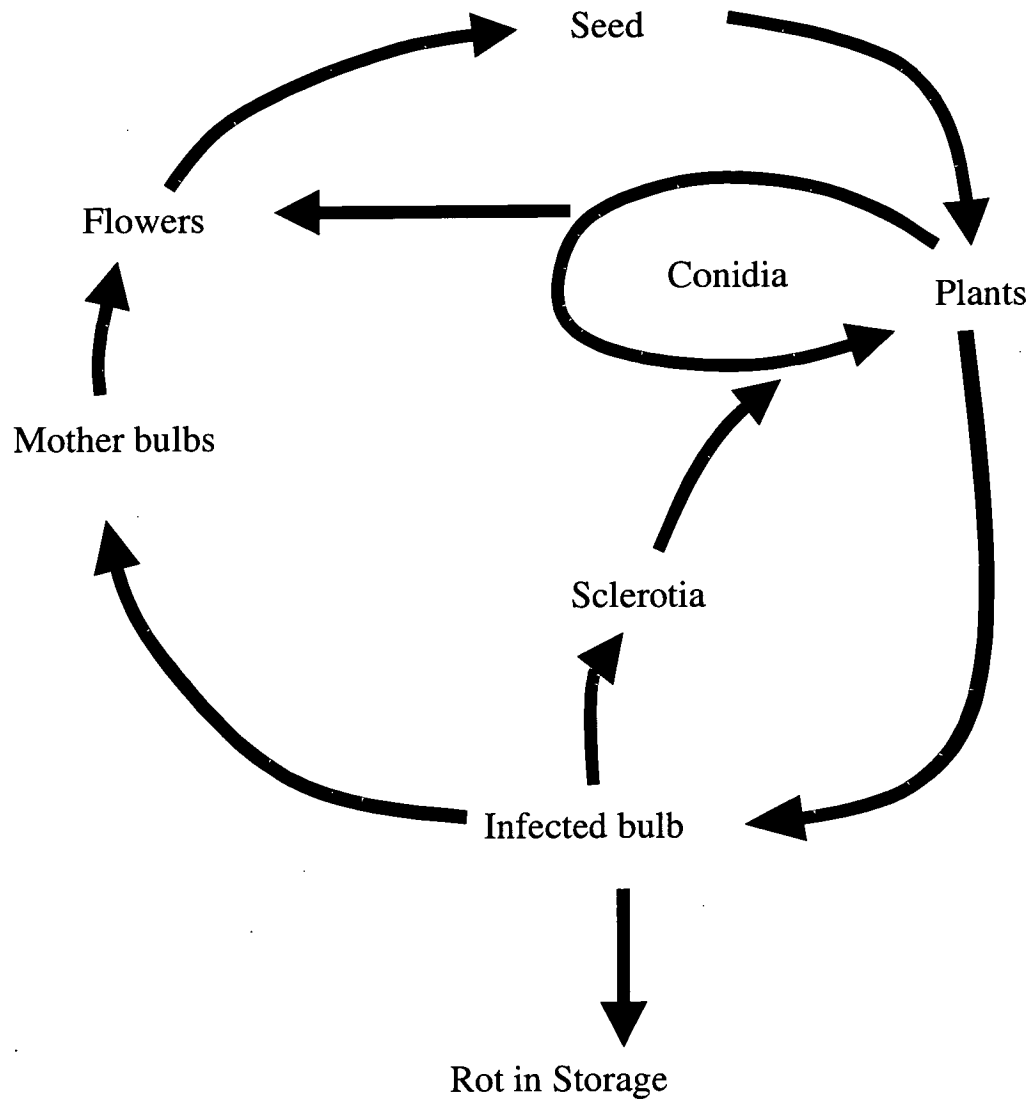

Figure 1.3: Life cycle of *B. allii* in onion (Adapted from Kritzman, 1983a).

1.4.3 Infection

1.4.3.1 Spore germination

Temperature

The optimal temperature for germination of *B. allii* conidia was found by Kritzman *et al.* (1980) to be 25°C. The time required for spore germination is relatively short. At

25°C, 30%, 63% and 95% of spores had germinated after 3.5, 5.5 and 7.5 h of incubation, respectively, on water agar (Kritzman *et al.*, 1980).

Kritzman *et al.* (1981) reported the optimal conditions for conidia of *B. allii* to penetrate into host leaves were 48 h of light, at 15°C and 98% relative humidity (RH). However, only 21.7% of the conidia penetrated the leaf surface, although the germination potential of the inoculum was 96% on media without nutrients (Kritzman *et al.*, 1981).

Humidity and leaf wetness

Blakeman (1973) reported many fungal pathogens can infect leaves from spores in a drop of rainwater or dew, as the fungi usually require free water or above 95% RH to germinate and infect a plant. The droplet of water is often referred to as an "infection drop".

Kritzman *et al.* (1981) found that *B. allii* penetrated leaves at 93-100% RH but not at relative humidities of 80-85% or 70-75%. Snow (1949) reported that *B. cinerea* required humidities above 90% to germinate, and that spores took 2 days to germinate at 93% compared to 1 day at 100% humidity.

One of the difficulties with experiments designed to measure the effect of humidity on spore germination is preventing condensation and, thus, free water on surfaces. Very slight changes in temperature to a system maintained at high humidity will lead to droplet formation. An atmosphere greater than 90% RH will be cooled below its dew point by a temperature drop of 1°C (Schein, 1964). These temperature changes occur

cyclically in incubators. Schein (1964) suspected that almost all work conducted in the range of 90-100% RH is subject to this artefact, and therefore spore germination recorded at high humidity may actually occur in free water and not in a near-saturated atmosphere. To overcome this problem, chambers can be submersed in a heated refrigerated Warburg apparatus bath, where temperature control is achievable within plus or minus 0.1°C or better (Schein, 1964). Kritzman *et al.* (1981) did not mention the accuracy of humidity control gained in their study. However, they reported a range of 93-100% RH which suggests there was fluctuation in the system and, most likely, condensation. Therefore, infection may have occurred in free water rather than high humidity *per se,* which illustrates that the infection droplet may be an important factor in plant infection by *B. allii.*

Few field studies have been conducted to examine the effect of environment on *B. allii* epiphytotics. Using bulbs from the same source planted at two different sites, a difference in disease (neck rot) incidence occurred (Kritzman, 1983). Disease was worse at the non-irrigated cooler site than at the warmer irrigated site. No explanation is offered by Kritzman (1983) for this apparent anomaly. However, perhaps there were different levels of inoculum pressure at each site, or weather conditions such as dew periods differed between sites.

Light

Germination of *B. allii* on water agar occurred with and without light (Kritzman *et al.*, 1981). However, better penetration of the onion leaf occurred with a light source than without, and at a range of temperatures. For example, fungal penetration was observed on the upper leaf tip (2-15 cm from the tip) at 15°C in three 48 hr light/dark regimes:

48 h of light; 24 h of light and then 24 h of dark; and 48 h of dark, where 22%, 21% and 2% of the spores germinated and penetrated the onion leaf surface, respectively (Kritzman *et al.*, 1981).

Germination inhibitors

A germination inhibitor was found to be produced from the spores of *B. allii*, which prevented them from germinating (Kritzman *et al.*, 1980). This is thought to be a strategy to avoid germination on the conidiophore or in situations with high spore concentrations.

Exogenous nutrients

No work has been published on exogenous nutrients and their effect on germination of *Botrytis* spp. involved in neck rot *per se*. However, there are records of *B. allii* possessing a high germination rate on media without nutrients (Kritzman *et al.*, 1981). Work has been published on related *Botrytis* spp. *B. cinerea* and *B. fabae*, which produce short germ tubes when dry conidia are placed on substrates at high humidity (Cole *et al.*, 1996). When spores were placed on a substrate in an aqueous glucose suspension, the germination tubes produced were long (Cole *et al.*, 1996). Further to this, some researchers have noted that spores of *B. cinerea* would not germinate in distilled water, but did germinate in a sucrose solution (Chou, 1972; and Clark and Lorbeer, 1977). However, Clark and Lorbeer (1977) reported that germination of *B. squamosa* (Walker) conidia was nutrient-independent.

1.4.3.2 Seed transmission of *B. allii*

Seed is potentially a very significant source of *B. allii* in an onion crop. Maude and Presly (1977a and 1977b) found that in the majority of cases, mycelium of *B. allii* grew from the seedcoat into the living tissue at the tips of the cotyledons during germination. Stewart and Franicevic (1994) also demonstrated that *B. allii* was able to pass from the seed to the resultant seedling, through the same process as described by Maude and Presly (1977a and 1977b).

Maude (1983) noted that during dry growing seasons, there was almost a one-to-one direct linear relationship between the percentage-infected onion seeds sown and the percentage of bulbs with neck rot. In wet humid growing seasons, the incidence of bulbs with neck rot was proportionally greater (Maude, 1983). This demonstrated how important seed can be as a source of infection for neck rot. Maude and Presly (1977a) and Stewart and Franicevic (1994) reported that occasionally the level of *B. allii* infection detected in the seedlings was lower than that found in the seeds. No reason was suggested by either researcher for this phenomenon.

1.4.3.3 Plant infection/ leaf penetration

Kritzman *et al.* (1981) found that most of the onion leaf penetration by *B. allii* occurred in the 2-15 cm region of the leaf from the tip. This region was approximately 10 times more susceptible than the region 15-35 cm from tip. Maude and Presly (1977a) also noticed that the leaf tips were infected more often than other parts of the plant. Neither of the researchers offered an explanation for this phenomenon. Perhaps it is associated with thinner or damaged leaf waxes at the leaf tip.

Kritzman *et al.*, (1981) found that infection of the onion leaf by *B. allii* occurred through stomata or directly through the epidermis. However, the germ tubes were not significantly attracted to the stomata. When the germ tube penetrated the leaf surface, it did so by either penetrating between two epidermal cells or directly into a single epidermal cell (Kritzman *et al.*, 1981). No anchoring point or appressoria were found (Kritzman *et al.*, 1981). In contrast, Tichelaar (1967) reported penetration only through stomata. He also reported that this process was preceded by the formation of an appressorium, and infection was through penetration of the guard cells, adjacent epidermal cells and the sub-stomatal cavity. Segall and Newhall (1960) did not find any direct penetration of germinated *B. allii* spores into leaves, but through stomata. There is, therefore, conjecture as to the actual method of plant infection by the fungus *B. allii*.

Tanner and Sutton (1981) studied the effect of leaf wetness and temperature on lesion development by *B. squamosa* on onion leaves. Temperatures of at least 12°C with periods of leaf wetness from 12-24 h were required for lesion production. Lesions were most frequent at 18°C with 24 h of leaf wetness, the longest leaf wetness treatment evaluated in this experiment (Tanner and Sutton, 1981). Alderman and Lacy (1983) dusted dry conidia of *B. squamosa* onto onion leaves and found lesions were initiated after a minimum of 6 hours of dew. Preliminary experiments by Alderman and Lacy (1983) showed that inoculating onions with aqueous spore suspensions gave variable infection results, but when applied dry through a spore settling tower, results were more consistent.

1.4.3.4 Fungal enzymes

During the first 19 hours of spore germination, Kritzman *et al.* (1981) found the enzymes cutin methylesterase, poly-galacturonase, and polyphenol oxidase to be secreted constitutively by *B. allii*. These enzymes degrade the cutin, the middle lamella, and the cell walls, thus enabling the pathogen to penetrate and establish in the host tissue (Kritzman *et al.*, 1981). Mayama and Pappelis (1977) concluded that *B. allii* penetration of the cell wall involved secretion of enzymes by the pathogen, which acted locally at the penetration site.

1.4.3.5 Quiescence and Pathogenesis

Walker (1926) considered *B. allii* to be weakly pathogenic on onion foliage. Tichelaar (1967) considered *B. allii* to be quiescent in the green leaf epidermis, only invading the leaf mesophyll tissue when the leaf senesced. Bochow and El Mosallamy (1979) also reported latent infections of green leaf and juvenile meristematic tissue. Senescent tissue was found to be colonised more often than non-senescent tissue. However, Maude and Presly (1977a) demonstrated that *B. allii* moves within green leaf tissue. They suggested two types of plant infection: 1) continuous infection throughout the whole plant, which originates from infected seed and develops from the base of each successive leaf that develops; and 2) the other more common form of discontinuous infection of leaf tips with subsequent growth of the fungus down the leaves.

Page (1955) could not get *B. allii* to induce leaf necrosis in the glasshouse, either by inoculating the surface with inoculum or by injecting the inoculum into the leaf blades. However, onion blast and leaf spotting were reported by Segall and Newall

(1960) to be caused by *B. allii*. Tichelaar (1967) failed to find *B. allii* causing leaf spots, contradicting the results of other workers (Hancock and Lorbeer, 1963; Segall and Newhall, 1960). Mayama and Pappelis (1977) reported that penetration of the outer epidermal cells at the base of onion leaves by *B. allii* caused a halo formation visualised with cell wall stains around the infection site. Stewart and Mansfield (1984) noted an effect of varying the inoculum concentration. At low inoculum concentration (less than 10^5 conidia per ml), *B. allii* failed to colonise tissues and was associated with the production of reaction material by epidermal cells of the plant. However, at inoculum concentrations greater than 10^5 conidia per ml, *B. allii* established distinct infection hyphae. Stewart and Mansfield (1984) suggest that the loss of virulence associated with the low spore concentrations may be caused by the loss of endogenous nutrients into surrounding water droplets, which may decrease rates of enzyme synthesis during the critical early stages of growth of infection hyphae. The production of reaction material thus prevents the invading fungus from killing epidermal cells.

1.4.4 Effects of environment on spore and sclerotia production

Conidia of *B. allii* tend to be larger and more infective when produced at lower temperatures. Cultures incubated at –2°C on potato dextrose agar (PDA) produced conidia 2.8 times larger in volume than conidia from cultures incubated at 20°C. The larger spores were quicker to germinate at low temperatures, particularly at -2°C and –4°C (Tian and Bertolini, 1996).

Honda and Mizumura (1991) investigated conidium formation under near ultra-violet light and continuous darkness. The number of conidia formed in darkness relative to

near ultra-violet light was greatest in *B. allii,* followed by *B. cinerea. Botrytis fabae* did not produce conidia under continuous darkness.

An antagonistic relationship exists between conidium formation and sclerotium formation, as sclerotia are formed under conditions that are unfavourable for conidium formation (Honda and Mizumura, 1991)

Clarkson *et al.* (2000) found temperature and water potential to affect the production of conidia by sclerotia of *B. squamosa.* The greatest numbers of conidia were found to be produced from 5 – 10°C, while at temperatures above 20°C few sclerotia produced conidia.

1.4.5 Spore release

1.4.5.1 *Botrytis* spore trapping

The majority of spore trapping work reported in the literature for *Botrytis* spp. is for *B. squamosa* and *B. cinerea.* The following table contains the dimensions of conidia of several *Botrytis* spp. pathogenic on onion:

Table 1.1: Dimensions of conidia produced by *Botrytis* spp. pathogenic to onion (derived from Hickman and Ashworth, 1943)

Species	Mean conidia length (μm)	Mean conidia breadth (μm)
B. allii	7-11	5-6
B. cinerea	8-14	6-9
B. squamosa	15-21	13-16
B. byssoidea	10-14	6-9

Little information is present in the literature on spore trapping of *B. allii*. Maude and Presly (1977a) used a Burkard Volumetric spore trap in infected onion crops in 1972 and 1973, but failed to trap air-borne *Botrytis* spores, even though conidiophores of *B. allii* were observed in the crop. Stewart and Franicevic (1994) reported that spore traps set out in field experiments also failed to detect *B. allii*, although *B. byssoidea* and *B. cinerea* conidia were routinely detected. No mention was made of the method of trapping or spore identification. However, in Germany Bochow (1981) reported that during July and August considerable numbers of *B. allii* conidia were trapped using a Rach'sche volumetric trap, with the spores differentiated by microscopic examination.

1.4.5.2 *Botrytis squamosa* spore trapping

Lorbeer (1966) used a Hirst spore trap and found that the release of *B. squamosa* conidia followed a diurnal pattern. The maximum numbers of conidia were caught in daylight hours during times of increasing temperatures and decreasing relative humidities. Lorbeer (1966) noticed that during one 36-hr period of continuous high RH (97%) the same diurnal periodicity of spore release occurred. Small (1970) indicated that fluctuations in RH appear to be involved in the liberation of *B. squamosa* conidia, through causing hygroscopic movements of conidiophores. Swanton (1977) also commented that rapid changes in RH and temperature, or a rain shower, were responsible for promoting spore release by *B. squamosa*.

Sutton *et al.* (1978) conducted spore trapping experiments with *B. squamosa* using a Burkard trap. They concluded that "the evidence indicated that the release of *B. squamosa* spores was promoted largely by declining RH and by rain." Lacy and

Pontius (1981) took the parameter of RH another step using a Burkard spore trap to trap conidia of *B. squamosa*. Vapour pressure deficit was found to be the most closely correlated environmental parameter with *B. squamosa* spore release, compared to temperature, RH, rainfall, leaf wetness, and wind speed and direction. Vapour pressure deficit (VPD) is calculated based on RH (VPD = saturated vapour pressure – ambient water vapour pressure at temperature T). The correlation between VPD and numbers of trapped conidia was most closely correlated (r = -0.67) at the temperature range of 10-24°C. Lacy and Pontius (1981), stated that temperatures outside 10-24°C inhibited spore release. The greatest number of spores were trapped on days preceded by two or more days of consistently low (0-3 millibars) VPD. The highest spore numbers occurred between 8 am and 1 pm. Spore catches were predicted with good accuracy using a table of estimated probability indices of spore release based on mean VPD and air temperature for the preceding three-day period (Lacy and Pontius, 1981). In a later paper, Lacy and Pontius (1983) again identified temperature and vapour pressure deficits as the weather variables most strongly associated with spore release. They again concluded that vapour pressure deficit correlated better with spore release than RH.

1.4.6 Spore dissemination

Campbell (1999) discussed 'botanical epidemiology', the primary goal of which is to understand how epidemics develop and what factors are critical to such developments. By understanding this, management strategies can be developed more effectively. Campbell (1999) noted that the way in which propagules are dispersed and the distances over which they are disseminated determines the character of an epidemic, the geographical scale of concern for the epidemic and ultimately the options for

disease management. An aerobiological view of the disease epidemic cycle can be broken into three sections: spore take-off, transport and deposition (Aylor, 1990). Release of spores under dry conditions can be divided into two categories: (a) actively released, or shed under the force of gravity and (b) those that are removed by fast turbulent wind (Aylor, 1990). Type 'a' release mechanisms are characteristic of fungi such as the basidiomycetes which actively disperse basidiospores. The release mechanism of downy mildews are also characteristic of type 'a' and are usually strongly correlated with other weather factors such as humidity and temperature. The type 'b' release mechanism includes fungi such as rusts and the *Botrytis* spp. where spore removal is often correlated with wind conditions. Aylor (1990) does point out, however, that the distinction between the two types of spore release mechanisms is not always clear and there can be significant dispersal of spores by other mechanisms such as rain splash.

Botrytis conidia often detach from the conidiophore in clusters, which results in a greater settling speed and higher rates of deposition near the source than single conidia (Harrison and Lowe, 1987). Harrison and Lowe (1987) demonstrated that the wind speed needed to be above 1-2 m/sec for significant numbers of spores to be removed from the conidiophore of these fungi. Turbulent wind is far more likely to penetrate and disrupt the boundary layer of air in a crop canopy and result in the removal of spores (Aylor, 1990).

Spore size and shape has been reported to have an influence upon the gradient of deposition for rain-splashed pathogens. Jenkinson and Parry (1994) reported that the *Fusarium culmorum* and *F. avenaceum* have different deposition gradients. *Fusarium*

avenaceum has macroconidia of 40-80 μm × 4 μm (length × diameter), which are larger than those of *F. culmorum* (15-20 μm × 5 μm). Furthermore, macroconidia of *F. avenaceum* are often clumped together. *Fusarium avenaceum* conidia were shown to be dispersed over shorter ranges than the smaller-spored species. A much larger splash droplet was thought to be required for dispersal of *F. avenaceum* vs. *F. culmorum* and, as such, would account for the shorter range of dispersal of the former species.

Attempts by Maude and Presly (1977a) in 1972 and 1973 to trap *B. allii* air-borne spores with a Burkard Volumetric spore trap were unsuccessful, even though conidiophores of *B. allii* were observed in the onion crop. Evidence for the distance of spread of *B. allii* was gained from uninfected plots of onion which had been sown 1.5 m and 270 m away from a plot sown with infected seed with a 20% incidence of *B. allii*. In the plot sown with infected seed, the incidence of infected plants increased to 70-75% after four months. The clean onion plot that had been established 270 m away, became infected with *B. allii* and reached an incidence of 4.6% after 4 months. The incidence of infection in the plot 1.5 m from the infected plot increased rapidly to 26.0% after 4 months. Maude and Presly (1977a) concluded that the spores of *B. allii* were windborne, as they observed disease spread within plots and from plots established with *B. allii*-infected seed to plots established with clean seed. In another experiment, they noted inoculum might have spread aerially from an infected plot to a healthy plot at a distance of 137 m (Maude and Presly, 1977b). From this, Maude and Presly (1977a) stated that "*B. allii* is thus source specific and not ubiquitous in onion crops and restricted in its range of spread to a distance of about 270m".

There are numerous reports of *Botrytis cinerea* being transmitted by thrips (Fermaud *et al.* 1994), *Drosophila* spp. (Louis *et al.* 1996) and moths (Bailey *et al.* 1997). Onion thrips (*Thrips tabaci*) are a common pest of onion in Tasmania. If it is possible for thrips to carry *B. cinerea* then it is highly feasible that onion thrips or other insects within the onion crop may disseminate *B. allii* conidia.

1.4.7 Conidia longevity/survival

Wagoner (1983, in Rotem *et al.*, 1985) stated that "all the words about the aerial dispersal of plant pathogens are wasted if the propagules are dead on arrival". Little literature exists on the length of survival of *B. allii* spores in the environment. Reports of spore survival for the species of *Botrytis* are variable. In greenhouse tests, Segal and Newhall (1960) found *B. allii* spores sprayed onto leaf surfaces retained their viability or at least capability to cause spotting for 8 days after application. The longevity of *B. cinerea* was found to be 3 minutes when exposed to ultraviolet (UV) light (Rotem and Aust, 1991).

Dry spores of *B. squamosa* applied to leaf surfaces survived 2 days with no reduction in inoculum potential on leaf surfaces, but viability declined after more than 2 days in a growth cabinet chamber at 60% RH (Alderman and Lacy, 1983). There was a 71% loss in infectivity of conidia by day 6 (Alderman and Lacy, 1983).

Harrison (1983) found the infectivity (potential to infect field bean leaves) of *B. fabae* spores decreased more quickly at 22°C than at 10°C. For example, the infectivity of conidia stored at 10°C/92% RH was halved after 34 days as opposed to storage at 22°C/92% RH, when infectivity was halved after just 3 days. In addition, low

humidities decreased the duration of infectivity. For example, at 92% and 42% RH the infectivity of spores was halved after 34 and 2.5 days, respectively. Harrison (1983) also found that conidia on spider webs declined in infectivity with time, with 15% of spores infective after 10 days exposure to ambient weather. Conidia survival is also affected by loss of UV-absorbing compounds, which occurs when *B. fabae* spores are soaked in water (Harrison, 1983).

Survival of conidia under short UV wavelengths, which are absent at the earth surface, was only 3 minutes for *B. cinerea* (Rotem and Aust, 1991). When spores were aggregated, the conidia survived much longer. When spores of *B. cinerea* were kept in darkness at a temperature of 45°C with a RH of 48.5%, germination was 30% after 1 day and 0% after 4 days. Few *B. cinerea* conidia survived when exposed to sunlight for a 12 hour period, reflecting the results found with UV light. Sclerotia of *B. cinerea* survived up to 5 days when placed in sunlight for 12 hour periods (Rotem and Aust, 1991).

The half-life of *B. cinerea* conidia attached to pear fruit in the field was found to be 14.4 days (Spotts, 1985). On pear fruit in the laboratory, conidia of *B. cinerea* survived longer at higher RH at the same temperature. The half-life of conidia in the orchard was found to be about 20-40% of that at similar temperature and RH in the laboratory (Spotts, 1985). Spotts (1985) cited Savulescu and Tudosescu (1968), who reported that *B. cinerea* was extremely sensitive to UV light and exposure of conidia to UV light for more than 2-hours reduced viability.

1.4.8 Sclerotia and *Botrytis* propagules in soil

Sclerotia are formed by *Botrytis* spp. and are generally considered to be the most important structures in the survival of the species (Coley-Smith, 1980). Sclerotia consist of a compact mass of hyphae, usually with a melanic rind (Agrios, 1997). The sclerotia of *B. squamosa* have been reported to survive in the soil at a depth of 15 cm. Ellerbrock and Lorbeer (1977a) reported 79% of sclerotia were still viable after 21 months in the soil. At lesser depths the sclerotia did not survive as well. For example, at a 3 cm depth only 16% survived after the same period (Ellerbrook and Lorbeer, 1977a).

Maude *et al.* (1982) found that sclerotia of *B. allii* were capable of surviving in sterile soil for a year before the viability began to decline, with some remaining viable for more than 3 years. However, in unsterile soil Maude *et al.* (1982) found sclerotia lose their viability within 6 months.

Ellerbrock and Lorbeer (1977a and 1977b) developed a selective medium for isolation of *B. squamosa* conidia in soil, which consisted of Martin's rose bengal agar (Martin, 1950) + amendments. This medium has also been used for the isolation of *B. allii* (Lorbeer and Tichelaar, 1970). *Botrytis squamosa* was isolated from field soil using soil dilution plating onto Martin's rose bengal agar plus amendments, during *Botrytis* leaf blight epidemics. However, *B. squamosa* was not recovered two weeks after harvest of the onions (Ellerbrock and Lorbeer, 1977a). Nonetheless, conidia were recovered from a field plot that was artificially infested with *B. squamosa* conidia, for up to 2 months from the time of infestation (Ellerbrock and Lorbeer, 1977a).

An increase in temperature reduced the length of survival of *B. squamosa* conidia in the study by Ellerbrock and Lorbeer (1977a), who noted that the optimal survival of conidia occurred at 3°C with survival progressively reduced at 21°C and 30°C, respectively.

In the same report, soil that was moist at the beginning of a laboratory experiment and then allowed to dry, showed the greatest reduction in conidia viability, compared with treatments where the soil was maintained either dry or moist (Ellerbrock and Lorbeer, 1977a). Little difference was observed between these latter two treatments. Ellerbrock and Lorbeer (1977a and 1977b) concluded from their experiments that *B. squamosa* does not survive over winter as mycelia in plant residue. However, cull piles and seed production fields were considered to be important sources of primary inoculum for initiation of *Botrytis* leaf blight epidemics in commercial onion fields in Orange County, California in the USA.

1.4.9 Seed as a source of inoculum

Maude and Presly (1977b) reported that *B. allii* was not ubiquitous but occurred where infected seed had been planted, unlike the ubiquitous pathogen *B. cinerea*. A direct link between infected seed and rotted bulbs was demonstrated by Maude and Presly (1977b), i.e., 1% infected seed resulted in 1% rotted bulbs in store. The direct relationship between seed infection and neck rot in store only held true under growing conditions in which weather was not conducive to disease development and spread. For example, in drier seasons, Maude and Presly (1977b) showed that the levels of neck rot corresponded to the percentage of seed infection. However, very wet summer conditions were very conducive to the development and secondary spread of the

pathogen within an onion crop. In the wet season, seed with an incidence of infection by *B. allii* of 1% gave rise to crops (planted at different sites) with 9% and 15% neck rot.

Stewart and Franicevic (1994) noted that not all seed infections were transmitted to seedlings. This is at odds with the result of Maude and Presly (1977b) who reported a 1:1 infection rate between seed and seedlings. Stewart and Franicevic (1994) evaluated seed transmission in three seed lots with an incidence of *B. allii* seed infection of 0.0, 10.3 and 30.3%, determined by first surface-sterilising the seed in 1% sodium hypochlorite for 2 minutes, followed by a rinse in sterile distilled water, and then plating 25 seeds per Petri plate containing Medium X agar (Last and Hamley, 1956) supplemented with streptomycin sulphate (30 µg/ml) and neomycin (30 µg/ml). The seed was treated with Captan (8 g active ingredient/kg seed) and sown in a commercial onion growing area. Commercial management practices and spraying regimes were used throughout the crop growth. The three seed lines, which initially had yielded 0.0%, 10.3% and 30.3% incidence of *B. allii* in the seed, resulted in 0.5%, 3.2% and 10.2% bulb rot, respectively, approximately 1/3 of the initial incidence of seedborne infection detected.

Maude and Presly (1977a) found that when seed with an initial incidence of *B. allii* of 20% was stored at 10°C and 50% RH the incidence of *B. allii* recovered from the seed fell to 3% within a year. When the same seed sample was stored at 22°C and 48% RH there was less than 0.5% incidence of seedborne *B. allii* after one year. Bochow (1981) also reported similar findings with a decline in *B. allii* infection of seed stored at room temperature. However, Maude and Presly (1977a) found the fungus was still

viable on seeds following storage durations of 3 years, which resulted in declining germination rate of the seeds (from a germination rate of 95% at the outset to 44% at 22°C and 74% at 10°C after 3 years). Hence, seed storage as a means of eliminating *Botrytis* infection of onion seed is not a viable option.

1.4.10 Transmission of *B. allii* from seed to plants

Maude and Presly (1977a) demonstrated that seed treated with Chloros (3% free chlorine) for 30 seconds reduced the level of detectable *B. allii.* However, the treatment did not eliminate *B. allii*, thus they concluded that a high proportion of infection was internal. Stewart and Franicevic (1994) conducted similar experiments in New Zealand. They found that recovery of *B. allii* from onion seed that was surface-sterilised with 1% sodium hypochlorite for 2 minutes was no different from the recovery of *B. allii* from seed washed in sterile distilled water. Therefore, they postulated that *B. allii* resides within the seed coat and not on the outside of the seed coat. Metcalf (1998) demonstrated that the level of seed borne infection could be substantially reduced by soaking seed in a solution of 10% hypochlorite. Soaking of infected seed for 1 minute in 1% NaOCl or 20 minutes in 10% NaOCl resulted in 6.7% and 0.3% of seeds giving rise to *B. allii,* respectively, when plated onto agar. This suggested that the pathogen may reside inside of the seed coat and can be killed if the seed coat is soaked in hypochlorite for sufficient time for the disinfectant to soak through the seed coat.

Maude and Presley (1977a) reported *B. allii* was transmitted from seed to seedlings by the fungus invading the tip of the cotyledon leaf from the attached seed coat (Table 1.2). They reported the mycelium then proceeded downwards in the cotyledon leaf,

only producing conidiophores when the leaf tissue became senescent. Occasionally they found that infection would move from the moribund cotyledon up into the first true leaves. However, more often, conidia production occurred on the cotyledon, leading to subsequent infections from the tops of the true leaves down (Maude and Presly, 1977a). Stewart and Franicevic (1994) also found that *B. allii* infected the cotyledon before it moved down the plant to infect the true leaves.

Table 1.2: Study of seed-to-seedling transmission, from Maude and Presly (1977a)

Method of identification	Days from sowing	% seedlings infected	% seedlings infected	
			Through the cotyledon tip	Elsewhere on the cotyledon and first true leaf
Staining	12	18	16	2
	21	39	37	2
	30	33	22	11
Isolation	30	32	18	14

1.5 Spore trapping

Spore trapping has been utilised by researchers for determining at what time of the season fungal spores are released and for identifying environmental conditions conducive to spore release. This information has been used to develop weather-based models for predicting spore release and improving the timing of implementing control strategies.

1.5.1 Spore traps

A number of spore traps are available for trapping fungal spores. Traps can be divided into the following general categories: sedimentary samplers, impingers and impactor traps.

Sedimentary sampling - the 'gravity slide'

Glass slides coated with a sticky substance, such as petroleum jelly or vacuum grease, can be used as a simple spore trap. If the slide is placed in a horizontal orientation, then spores may settle on the surface through gravitation. However, if the slide is placed at an angle of 45° and faced into the wind, then spores will also be impacted onto the surface (Campbell and Madden, 1990). The sampling efficiency of glass slides is low and greatly influenced by wind speed.

Impingers

Impingers work by passing an air sample through a fluid and trapping spores in the fluid (Davies, 1971). This form of trapping is especially useful for trapping microbes that can be dilution plated onto selective media for enumeration. It is particularly useful for trapping bacteria (Campbell and Madden, 1990).

Impactors

The impactor type of spore trap is used widely for fungi (Davies, 1971). The rotorod type works by impacting airborne particles on a rotating surface. Other impactors, such as the Burkard trap (Burkard scientific sales limited, Rickmansworth, Hertforshire, England), make use of an air pump to impact air onto a stationary or slowly-rotating surface. The Burkard trap uses a clear (Melinex) tape fitted to a drum

that is slowly rotated past an inlet orifice through which air is sucked by means of a pump. The tape is later mounted onto a glass microscope slide and examined under a microscope for visual identification of the spores. The trap can operate for a week before the tape needs changing. This allows spores to be trapped and enumerated over week-long periods. Another common type of impactor trap, the Hirst trap (Hirst, 1952), also uses a pump to draw in air. However, spores are trapped on a slide which is moved past the inlet orifice. Hirst (1953) discussed his spore trap and general patterns of spore and pollen release. He also reported on the efficiency of horizontal slides when used at varying wind speeds. Small (1970) used a Hirst spore trap for continuous sampling of air for his studies on *B. squamosa*.

Gadoury and MacHardy (1983) described an inexpensive PVC spore trap alternative to the Burkard spore trap. Wili (1985) defended the Burkard trap and mentioned some of the short falls of the PVC trap. In particular, Wili (1985) stated that changing the distance between the trapping orifice and the adhesive surface from 0.6 mm (Burkard) to 1.0 mm (PVC trap) greatly reduced the trapping efficiency, particularly spores with diameters less than 0.02 mm.

The Quest trap used in this thesis is manufactured by Melpat International (Quest developments, Brits, South Africa). It also has a distance of 1.0 mm between the inlet orifice and the trapping surface. However, the flow rate of the Quest trap is higher than the Burkard trap (10 L per minute) at 20 L per minute, which compensates for the greater distance between aperture and trap surface compared to the Burkard trap.

1.5.2 Fungal identification

In the past, two main methods have been used for identifying and quantifying fungal spores, culturing and visual identification. For the culturing techniques, the number of spores (or more correctly colony-forming-units (CFU's)) are determined by collecting fungal propagules onto Petri-plates containing selective agar media. The plates are incubated and the colonies counted to determine the number of CFUs from air samples. This method of identification has advantages for fungi with spores that are not distinctive. It also gives a measure of viability of trapped propagules. However, fast-growing contaminant fungi can limit the applicability of this technique. In addition, not all fungi can be cultured on agar and, for the fungi that can, some can be difficult to distinguish from similar fungi on agar. The other principal method of visual identification relies upon distinct morphological properties of fungal spores for their recognition, i.e., colour, size, and shape. The disadvantage with visual identification is that many fungi produce spores that are morphologically similar and some can be extremely difficult to visualise (Warner, 2002).

New methods are being developed for the identification and quantification of spores from air, soil, plant and water samples. These methods include the use of polymerase chain reaction (PCR) and immuno-detection procedures. PCR techniques can also be used for the identification and enumeration of fungal or aerial micro-biota (Hernandez *et al.*, 1999). PCR primers developed by Wiglesworth *et al.* (1994) were capable of detecting amounts of DNA (1-10 fg) less than that contained in a single spore of *Peronospora tabacina.* A real-time quantitative PCR method was demonstrated by Bohm *et al.* (1999) for the quantification of *Phytophthora citricola* DNA. Using quantitative PCR and specific primers, it may be possible in the future to determine

the amount of target DNA and, therefore, the number of specific spores of a particular fungus in a sample. Likewise, immunogenic procedures can be used to quantify the number of spores in a sample. Such techniques include the direct visual identification of spores through immuno-fluorescence microscopy, a technique used for *Pyrenopeziza brassicae* (Tijs Giles, *personal communication*). Monoclonal antibodies are generally used due to their high specificity and low cross reactivity. Kennedy *et al.* (2000) demonstrated a microtitre immunospore-trapping device (MTIST). The MTIST traps fungal spores by impaction of the spores into wells of polystyrene microtitre plates. Using monoclonal antibodies and enzyme linked immunosorbent assay, the number of ascospores or conidia of target fungi could be quantified using a standard calibration curve of absorbance versus the number of ascospores or conidia per micro-titre well (Kennedy *et al.*, 2000).

Immunological or PCR techniques for routine detection of small numbers of *B. allii* spores may assist in understanding the windborne spread of this fungus. Unfortunately, at present there are no monoclonal antibodies or specific polyclonal antibodies for use in identifying *B. allii* spores. Therefore, at present we have to rely on visual identification of spores or the use of culture techniques. Perhaps in the future monitoring techniques such as those described above may allow strategic control strategies to be used for *B. allii*.

1.6 Neck rot disease control/prevention

1.6.1 Seed testing

Maude and Presly (1977a) demonstrated that the use of disease-free seeds would assist greatly in *B. allii* disease prevention.

1.6.2 Seed treatment

Tichelaar (1967) found that treating infected seed with benomyl could reduce subsequent incidence of rot in store. The exact relationship between infected seed and rot in store was not described.

Maude and Presly (1977b) reported that the use of a benomyl seed dressing greatly reduced the level of resultant bulb rot originating from infected seed lots. However, treatment did not completely control disease. Whether disease had originated from the treated seed or had come from another source was unknown. Maude and Presly treated 10000 seeds with a 43% incidence of infection by *B. allii* with benomyl. The seeds were sown into compost in sealed plastic boxes. When the seedlings were 4 weeks old they were removed and incubated on moist tissue pads in closed plastic containers for 5 days before being assessed for conidiophore production. Only eight infected seedlings were recovered, demonstrating that the fungicide treatment did not provide absolute eradication. The recovered isolates of *B. allii* were found to be sensitive to benomyl. Thus, where infected seed is treated with benomyl and some infection is still detected in the crop, this may be due to a number of factors such as fungicide resistance, and/or lack of complete inhibition of the pathogen by the fungicide or incomplete application of fungicide to all seeds (Maude and Presly,

1977b). Bochow (1981) also stated that seed-borne primary infection of onion seedlings may not always be eliminated with fungicides.

Presly and Maude (1980) used carbendazim treatment of seed to delay the onset of *B. cinerea* infections of salad onion seedlings by 9 weeks and 3 weeks in each of two different seasons. However, after 3 seasons of use of the carbendazim seed treatment, isolates of *B. allii* were detected that were insensitive to carbendazim.

Metcalf (1998) demonstrated that the level of seed borne infection could be substantially reduced by means of soaking seed in a solution of sodium hypochlorite. For example, when a batch of seed was soaked for 1 minute in 1% sodium hypochlorite the incidence of *B. allii* recovered was 8.6%, when the seed was soaked in a 10% solution for 20 minutes the incidence of *B. allii* fell to 0.4% (Metcalf, 1998). Metcalf (1998) suggested that this method may be used as a seed treatment. When sodium hypochlorite-treated seed was sown in a field trial, no negative effect upon germination or plant growth was noted. However, the trial was not assessed for the incidence of plant infection or bulb neck rot (Metcalf, *personal communication*).

Mebalds *et al.* (1997) investigated the use of steam-air treatments to control seedborne microbial pathogens of a range of vegetable and flower crops. They demonstrated very effective control of *B. allii* on onion seed with this technique. The onion seed line used in the trial had an incidence of 17.3% external and 6.8% internal *B. allii* (tested by plating non-sterilised and surface-sterilised seed). Treatment of seed at temperatures ranging from 50°C to 60°C (with 2°C intervals tested between these temperatures) for 30 minutes, were shown to remove *B. allii* completely from the

seed. However, seedborne *Aspergillus niger* was not controlled at any of the temperatures evaluated. This may have been due to the thermophilic nature of *A. niger* (Mebalds *et al.*, 1997). The germination capacity of the onion seed was not significantly affected by the steam-air treatments. This demonstrated that steam-air treatments may be used to control some fungal pathogens, in particular *B. allii,* without any negative effects on seed quality.

1.6.3 Fungicides and benomyl resistance

Current commercial practice in the Tasmanian onion industry for control of *B. allii* is to apply Benlate (benomyl) at 1.0 kg/ha plus Folicur (tebuconazole) at 300 ml/ha. Fungicides are applied as two sprays 7 days apart at the 4-5 leaf stage followed by a further 2 applications 7 – 10 days apart at the commencement of bulbing.

Fungicide-resistant strains of *B. allii* have been reported overseas (Maude and Presly, 1977b). In Israel, Kritzman (1983) trapped *B. allii* spores on a selective medium and then tested isolates for sensitivity to benomyl in agar. He found that 90% of the isolates were resistant to benomyl in agar plates, but highly sensitive to iprodione and vinclozolin. However, the method of testing for benomyl resistance was not given in the paper.

Luck and Gillings (1995) developed a PCR test for *B. cinerea* isolates that could detect a single base pair change associated with benomyl-resistant isolates. They reported that the test might be useful for detecting fungicide resistance in other fungi.

1.6.4 Hygiene

Hygiene is another strategy that can be used to minimise losses from onion neck rot (Dennis, 1996a). Removal of volunteer plants and cull piles, which can be sources of inoculum, can reduce the level of initial inoculum around a crop. Crop rotation is also practiced in Tasmania, with onions only being planted in the same patch of ground 1 in 4 years, to reduce the likelihood of soil-borne *B. allii* inoculum (Maude *et al.*, 1982).

1.6.5 Cultural practices

The practice of artificial curing has been demonstrated by Maude *et al.* (1984) to reduce *Botrytis* bulb rot in store. However, the procedure only worked if the topped onions were harvested within 48 hours of topping and were dried at 30°C (with an airflow of 425m^3 air/hour/tonne). Onions dried at ambient temperature (18°C) had a greater incidence of onion neck rot than those dried at 30°C (Maude *et al.*, 1984).

Differences in susceptibility of onion cultivars to *B. allii* has been found, as was demonstrated by Ahmed *et al.* (1992). Rod (1996) stated the differences in cultivar susceptibility to neck rot, when grouped according to bulb colour, were not significant, although white cultivars were relatively more susceptible and red ones relatively less so than the brown cultivars.

1.6.6 Biological control

Walker and Maude (1975) demonstrated that *Clonostachys rosea* was a consistent contaminant of *B. allii* isolates taken from growing plants and onion bulbs. *C. rosea*

prevented sclerotia of *B. allii* from germinating and infected sclerotia were deeply penetrated by the fungus. On tests in agar cultures, Walker and Maude (1975) found that *C. rosea* produced substances which inhibited the growth of *B. allii* hyphae, and *B. allii* spores failed to germinate on agar when mixed with those of *C. rosea.*

Kohl *et al.* (1991) investigated biological control agents on onions artificially infested with *B. aclada (B. allii).* The investigation demonstrated that in field experiments with artificial inoculation with conidia of *B. allii*, applications of *Trichoderma viride* during harvest, reduced the incidence of *B. allii* neck rot assessed after 3 months storage, from 35% to 24%, and from 28% to 18%, in two separate years, respectively. However, the authors noted the difficulties in using biological control agents against *Botrytis* in commercial onion crops. As there is only a short period of time after the wounding before the pathogen becomes established in the host plant, the antagonists need to be actively present on the leaf surface in sufficient numbers whenever the wounds are made.

Kohl *et al.* (1995) demonstrated that the physical removal of necrotic onion leaf material from within a crop could reduce the level of *B. cinerea* spores in the air and also retard epidemics of onion leaf spot (*B. squamosa*). They concluded that applications of antagonists to suppress sporulation of *Botrytis* spp. on necrotic leaf tissue could be an effective control strategy. Kohl *et al.* (1997) found that the application of *Ulocladium atrum* completely excluded *B. allii* from dead onion tissue when both fungi were competing for the same substrate. However, no evidence of parasitism was found. Kohl *et al.* (1997) concluded that *U. atrum* could compete for

and utilise necrotic tissues more rapidly and extensively than *B. allii*, thereby excluding competitors without any antagonistic action.

To the author's knowledge, no biocontrol agents for use against *B. allii* are commercially available currently.

1.7 Fungal epidemiology and methodology

1.7.1 Disease survey methods

Various systematic sampling patterns can be used for sample collection in plant disease epidemiological studies (Figure 1.4). The sampling pattern used is dependent upon the information to be collected (Barnett, 1986). Systematic sampling patterns can be designed to sample for different types of spatial disease patterns. Pattern 'b' in Figure 1.4 is one of the simplest to perform in a field situation and would be suitable in a small paddock where no obvious disease gradients exist. Patterns 'c' and 'e' would be better than 'b' in large fields in order to cover more of the field. Patterns 'd' and 'g' could be used to give priority to edges of the sampling area if edge effects were being examined. The intensive pattern 'f' could be used to determine not only edge effects but also direction of spread of a pathogen (Barnett, 1986). Pattern 'e' would be the best pattern to determine the incidence of *B. allii* in a paddock, as paddocks are large, and this pattern provides a good coverage of such an area, without being as time consuming as a pattern 'f' which might be used if spatial information was being collected.

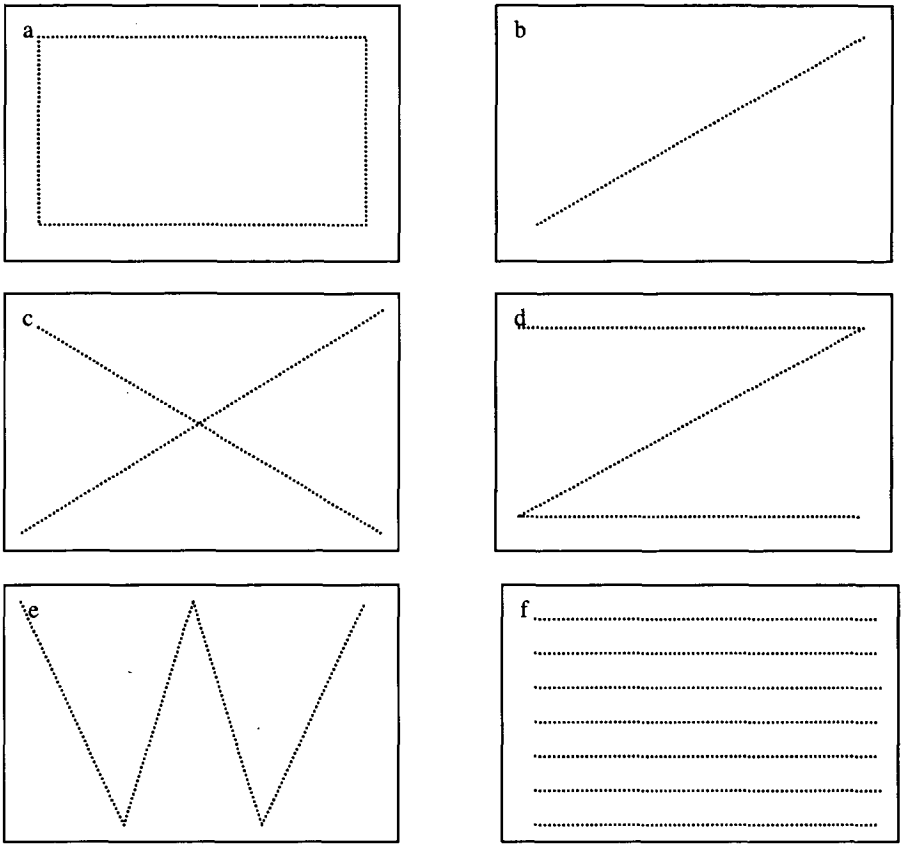

Figure 1.4: Sampling patterns for plant disease epidemiological studies (Barnett, 1986)

1.7.2 Disease progress curves

The plot of disease incidence with time, the disease progress curve, has been referred to as the "signature" of an epidemic. The disease curve represents all factors occurring during an epidemic, i.e., the interaction between pathogen, host and environment (Campbell and Madden, 1990). Characteristics of a disease epidemic can be investigated through analysis of disease progress curves; including time of disease onset, initial and final levels of disease, rate of disease increase, shape of the disease curve, maximum and final amount of disease and duration of the epidemic (Bowers and Kinkel, 1997). To the authors knowledge disease progress curve have not been used to analyse a *B. allii* epiphytotic.

The commonly-used temporal population growth models used to describe epiphytotics of plant pathogens are the monomolecular, exponential, logistic, Gompertz and linear models (Campbell and Madden, 1990).

The monomolecular model was originally used to describe the temporal dynamics of chemical reactions (Campbell and Madden, 1990). For example, certain enzyme reactions are fastest at the beginning of the reaction, but the rate of the reaction slows as the amount of free substrate available for pairing with enzyme molecules becomes limiting. As applied to plant disease epiphytotics, the model assumes that the rate of change in disease is greatest in the beginning and slows as the amount of available disease-free plant tissue decreases (Table 1.3).

The exponential model (Table 1.3) is also known as the logarithmic or Malthusian model (Campbell and Madden, 1990). It assumes that the absolute rate of disease increase is directly proportional to the amount of disease. Furthermore it assumes that host tissue is not limiting and can be used to describe the early stages of most polycyclic epiphytotics.

The Gompertz growth model (Table 1.3) is very similar to the exponential model in the early phases, where Y (the incidence of disease) is below 0.5-proportion. However, as plant tissue becomes limiting ($Y > 0.5$) then the rate of disease increase reduces. As for the logistic curve, the Gompertz curve has an inflection point. However, this occurs at $Y = 0.37$, much earlier than the logistic curve inflection point at $Y = 0.50$, and a greater proportion of the area under the disease progress curve is to the right of the inflection point, than to the left.

The logistic growth curve (Table 1.3) is similar to the Gompertz and exponential curves at the start of an epidemic, when low levels of disease are present (Campbell and Madden, 1990). However, the full logistic curve is sigmoid in shape, as opposed to the Gompertz and exponential curves which are not sigmoid. Biologically, as the available healthy plant tissue becomes infected in the progression of the disease, there is less tissue available for infection and the rate of infection falls (similar to the monomolecular model,) thus giving the sigmoid shape curve (Campbell and Madden 1990). The inflection point is at 50% disease incidence (Y = 0.5).

The linear model assumes the absolute rate of disease increase will be constant throughout the epiphytotic (Campbell and Madden, 1990). Therefore, the rate curve for the linear model approximates a horizontal line.

Table 1.3 : Growth models and their formulae (Campbell and Madden, 1990)

Model	Integrated equation	Absolute rate equation	Linearized equation
Monomolecular	y = 1-(1 - yo)exp(-r*t)	dy/dt = r*(1 - y)	ln[1/(1 - y)] = ln[1 / 1 - yo)] + r*t
Exponential	y = (yo)exp(r*t)	dy/dt = r*y	ln(y) = ln(yo) + r*t
Logistic	y = 1 / {1 + [(1 - yo)/yo]exp(-r*t)}	dy/dt = r*y*(1 - y)	ln[y/(1 - y0] = ln[yo / (1 – yo)] + r*t
Gompertz	y = exp({[ln(yo)]exp(-r*t)}	dy/dt = r*y*[-ln(y)]	-ln[-ln(y)] = -ln[-ln(yo)] + r*t
Linear	y = yo + b*t	dy/dt = r	y = yo + b*t

y = disease at time of observation

y_O = level of disease at first observation

r = rate of disease increase for a specific model

t = epidemic duration

b = constant increase in incidence of disease (r = 0)

For some plant disease epiphytotics, none of the above growth models may be appropriate for describing the epiphytotic, especially where there are fluctuations in *dy/dt,* i.e., where there is an irregular shape to the plot of disease incidence versus time. For the purposes of summarising a disease progress curve for comparative or analytical purposes, the area under the disease progress curve (AUDPC) can be used as a quantitative descriptor of an epiphytotic.

Botrytis allii is a polycyclic pathogen, meaning that it is capable of going through more than one generation per crop/plant season. With each disease cycle the inoculum increases many-fold. To the author's knowledge no data has been published on the possible increases in inoculum with each disease cycle.

Bochow and El-Mosallamy (1979) concluded that, as the growing season progresses, more senescent tissue becomes available on which for *B. allii* can sporulate. *Botrytis squamosa* has also been reported as a mid- to late-season pathogen (Small, 1970; Swanton, 1977), due to the increase in senescing onion tissue once bulb initiation occurs (Alderman and Lacy, 1984b). Thus exponential or logistic type disease models are likely to describe the *B. allii* disease progress, better than other growth models such as the linear growth model, which describes a constant increase in disease incidence

1.7.3 Spore deposition gradients and disease gradients

Disease gradients (i.e., decline in disease over distance) can result from a number of different causes (Campbell and Madden, 1990). Disease gradients can be caused by environmental gradients, such as microclimate changes, soil fertility changes or by inoculum dispersal gradients. Inoculum sources that cause disease gradients can be divided into three categories: point, line and area sources.

Disease gradients can be described by power law and exponential mathematical models (Table 1.4), where Y is the amount of disease and X is distance, usually from the source of disease. For both equations, the value of Y decreases with increasing distance X from source. The constant 'a' is numerically equal to the value of Y at X = 1. The constant 'c' is the value of Y at X = 0 (i.e., the source of inoculum). To enable comparison between disease gradients, the equations can be made linear by taking natural logarithms of both sides of each equation. The exponents 'b' and 'd' then function as the slopes of the linear equations of the power law and the exponential

model, respectively. The parameters 'a', 'b', 'c', and 'd' can be estimated from the linearized model plots (Table 1.4).

Table 1.4: Mathematical models used to describe disease gradients

Model	Model	Linearised model
Power law	$Y = aX^{-b}$	$\ln(Y) = \ln(a) - (b)\ln(X)$
Exponential	$Y = c \exp(-dX)$	$\ln(Y) = \ln(c) - dX$

Fitt *et al.* (1989) stated that the deposition gradients for spores borne in splash droplets were fitted better to exponential models than to power models, while fungi that had air borne spores less than 10 μm in diameter were fitted better to power law models. The models described above are empirical models. Fitt *et al.* (1989) stated that both of these empirical models have limitations and should not be extrapolated to distances outside the observed range. Spore deposition has often been modelled using either an inverse power law [$y = a x^{-b}$] or a negative exponential law [$y = a \exp(-bx)$] (Aylor, 1990). Rain-splash dispersal is well described by an exponential model (Fitt *et al.*, 1989). An exponential model should also describe dry-spore dispersal in situations where turbulence is slight and spores are mainly transported within the canopy (Aylor, 1987). In addition, there is another class of disease gradient models which are more complex, called physical models, including those adapted from meteorological pollution dispersal models (Aylor, 1990). Some physical models include the Gaussian plume model, the Gradient diffusion (K-theory) model and the Lagrangian random-flight simulation model (Aylor, 1990).

Ferrandino (1993) made the point that the net effect of spore escape from the canopy is a steeper dispersal gradient near the source of inoculum, where spores are carried away from the region of the canopy being monitored and, therefore, are not included in the measure of the disease gradient. Dispersal of a pathogen in a tall crop may result in a shallower disease gradient than a similar pathogen in a shorter crop, as spores are more likely to be trapped by the plant canopy in the tall crop than the shorter crop.

Use of disease gradients to monitor or characterise development of an epiphytotic has the advantage over the use of inoculum gradients as all of the processes that lead up to spread of the disease (i.e., the production, release, and transport of spores and plant infection by the spore propagule), have been accounted for. However, the use of inoculum gradients has the advantage of measuring directly the number of propagules transported to different distances (Mundt *et al.,* 1999).

Wu *et al.* (2001) reported that dissemination of plant pathogen propagules by water usually results in short range transport and the formation of steep disease gradients. Dissemination of spores by wind is thought to operate from a dual dispersal phenomenon. Some spores are transported within the canopy, resulting in shallow disease gradients, while other spores escape for longer-distance dissemination, leading to shallower disease gradients (Aylor, 1990).

1.8 Spatial analysis

1.8.1 Introduction to spatial analysis

Analysis of spatial patterns of disease can provide insights into the spatial characteristics of epiphytotics and allows the development of plausible biological and environmental hypothesis to account for the associations among pathogen propagules or diseased plants (Campbell and Madden, 1990). The analysis and description of spatial characteristics of pathogens and diseased plants is vital for modelling and simulation activities and for designing experiments and sampling programs for disease epidemiology and management studies (Campbell and Madden, 1990).

1.8.2 Ordinary runs analysis

Ordinary-runs analysis was first described by Madden *et al.* (1982) for detecting clustering of infected plants in rows. Ordinary runs analysis defines a run as "a succession of one or more plants of identical infection status followed and preceded by a plant of different infection status". The null hypothesis states that the ordered sequence of infected plants is random. The alternative hypothesis states that the ordered sequence of infected plants is not random. The number of expected runs, E(U), is based upon the number of infected plants in a row (m) and the total number of plants (N):

$E(U) = 1 + 2m(N-m)N$

The number of expected runs, E(U) is then compared to the number of observed runs, U. The observed number of runs will be less than the expected number if there is clustering of infected plants. A normal test is used to determine clustering. A Z-statistic less than –1.64 indicates clustering (P = 0.05):

$Z = [U - E(U)/s(U)]$

Ordinary runs analysis can only be used to determine if a run of plant infection status are random or non random. It is a simplistic method, however useful for testing for aggregation in the data set. It does however require that units are regularly spaced.

1.8.3 Beta Binomial Distribution

The beta binomial distribution (BBD) program (Madden and Hughes, 1994) tests for fit of a binomial and a beta binomial distribution to the test data set. A good fit to the binomial distribution suggests a random spatial pattern of the disease, while a good fit to the beta binomial distribution suggests an aggregated spatial pattern of disease incidence (Turechek and Madden, 1999). The beta binomial distribution has two parameters, p, which is the expected probability of disease and θ, which is a measure of the variation in disease incidence per sampling unit. The binomial distribution has a single parameter (π) representing the probability of disease. An index of dispersion (D) is produced by dividing the observed variance in incidence of diseased plants per sampling unit by the theoretical variance for a binomial distribution (Madden and Hughes, 1994). D is directly analogous to the standard variance-to-mean test for count data. The program gives outputs of the goodness-of-fit of both distributions and the maximum likelihood estimation (MLE) for each of the distributions. A log-likelihood ratio test statistic (LRS) can be used to determine if the beta-binomial distribution fitted the data better than the binomial distribution (Turechek and Madden, 1999). The LRS equation is:

LRS = 2 * (log-likelihood of the beta binomial distribution – log-likelihood of the binomial distribution)

LRS has a χ^2 distribution with 1 df under the null hypothesis of no difference between the two log-likelihoods (Turechek and Madden, 1999).

The BBD has limitations similar to that of the ordinary runs analysis, in that it only tests if a data set is aggregated, however it does so through a different set of calculations.

1.8.4 SADIE (Spatial Analysis by Distance IndicEs)

Spatial Analysis by Distance IndicEs (SADIE) was introduced by Perry and Hewitt (1991). Prior to SADIE, techniques to measure spatial patterns of counts were limited to descriptions of frequency distributions and analysis of variance-mean relationships.

SADIE quantifies spatial patterns by measuring the minimum total effort required to move individuals in the sample to either extreme uniformity (regularity, D) or aggregation (crowding, C). Alston (1996) argued that the distance to regularity, D, provided a better basis for an index to detect multiple clusters, clumps or patches than the distance to crowding, C. The distance to crowding (C) is only appropriate where there is a single focus. The degree of randomness is then quantified by comparing the observed spatial pattern with rearrangements in which the sample counts are randomly redistributed among the units (Perry, 1995ab).

By dividing the observed D with the average D from the randomisations, an index of aggregation, I_a, is calculated. An aggregated spatial pattern has an $I_a > 1$, whereas an $I_a = 1$ indicates a spatially-random pattern, and a regular spatial pattern is indicated by an $I_a < 1$ (Perry, 1998).

SADIE provides the coordinates of the centroid of the counts, which is analogous to the arithmetic mean. A useful statistic can be determined from the difference between the centroid of counts and the centroid of the sample units, δ. δ provides information on the displacement of the population from the centre of the sample area towards an edge. If counts are very close to the edge of the sampled area, δ values are large which, in turn, causes high I_a values. Perry and Klukowski (1997) revealed two methods to deal with these situations. The first is termed the translation technique, which basically moves the zero counts around the cluster of results so that the δ value is brought to zero. The second 'permutation' technique works by swapping the count values within pairs (i.e., not moving any of the zero values) to minimise the δ value.

Plots of the clustering indices provide local measures of aggregation and demonstrate where significant outflows and inflows occur to distribute the count data evenly. The plots were termed 'red-blue' plots (Perry *et al.*, 1999). Areas of relatively large counts close to one another were referred to as 'clusters' and were demonstrated in red. Areas of relatively small counts (gaps) were demonstrated in blue. Clusters may be defined using various levels of threshold index values (Perry *et al.*, 1999).

Perry and Dixon (2002) introduced a new method to assess the spatial association between two sets of count data. The method is based on a comparison of the spatial SADIE clustering index of two sets of data at each of the sample units. A randomisation method allows the construction of a test of critical values and the spatial association can be mapped, identifying clusters of units with positive association or negative dissociation (Perry and Dixon, 2002).

SADIE does not require the regular spacing of units unlike, ORA and BBD analysis. It also provides a measure of overall association for the data set, and a graphical output to determine the location of significant association or dissociation, thus providing advantage over the above spatial analyses.

1.8.5 Radial correlation analysis

Quadrat methods of measuring spatial distribution are insensitive to disease density changes on a scale larger or smaller than the quadrat size, and to spatial alignment of infected plants within quadrats (Gray *et al.*, 1986). Radial correlation analysis is based upon the calculation of the number of infected plant pairs within a certain range. It is an extension of two-dimensional distance class analysis, first developed by Gray *et al.* (1986) and then extended into the 2DCLASS program (Nelson, 1995).

Ferrandino (1996) reviewed two-dimensional distance class analysis. He demonstrated problems related to the Monte-Carlo simulation method for evaluating confidence limits. For a large field of 1000 plants with high disease incidence, the appropriate number of simulations needed for accurate statistics was demonstrated to be $4 * 10^5$, considerably more than the 400 recommended by Nelson *et al.* (1992). This is due to the large number of distances and angular orientations tested and, consequently, the appearances of Type I errors (false positives).

Radial correlation analysis accounts for differences between reference- and target-plant populations (Ferrandino, 1998). Radial correlation analysis calculates a fraction of the total number of infected plant pairs in every section. The technique judges correlations over and above any trends already present in the field, such as edge

effects or anomalous distributions. The observed and expected fraction of pairs at certain separation units between plant pairs (r), and the delta (difference between expected and observed) between pairs is plotted. Where the difference of these two curves is greatest, the deviation between the two curves is assessed at 'r' separation units. If the deviation between these two curves is significant at the 5% level, this is indicated by a one-tail Kolomogorov-Smirnov (KS) statistic. The critical delta value for the KS statistic is calculated from the infected number of plant pairs (n) to obtain the degrees of freedom (n=1) at the 5% level. The KS statistic is an objective method to define a length scale. The x position of maximum value defines the length scale of significance. KS type analysis is based on the cumulative probability density function for the total number of infected-infected plant pairs within a given distance. This method independently yields an estimate for the length scale over which disease is correlated and the probability of deviation from a random spatial distribution (Ferrandino, 1998; Gottwald *et al..*, 2002). The number of Type I errors (Type I: H_0 true, reject H_0, $P = \alpha$) are decreased by applying a Bonferroni correction factor.

Unlike all the spatial analysis methods mentioned above radial correlation analysis provides information on the distribution of plant pairs, showing aggregation of infected plant pairs in specified orientations against distance between the infected plant pairs.

1.9 Disease modelling

1.9.1 Fungal disease prediction models

Fungal disease models have been developed for the understanding of the biological processes of epiphytotics and for the management of these diseases. Examples include, among others, models for apple scab (Aylor, 1998) and onion downy mildew (Sutton *et al.*, 1986; Alderman *et al.*, 1987; Vincelli and Lorbeer, 1989; De Visser, 1996).

1.9.2 *Botrytis squamosa* models/forecasting

Shoemaker and Lorbeer (1977ab) developed a predictive model for the timing of control measures for *B. squamosa.* They used a critical disease level (CDL) of 1 lesion per 10 leaves to initiate fungicide applications. They also found that a Hirst spore trap could be used to detect spores two weeks before the CDL was detected in a crop, and suggested that the spore trap may be useful as an alternative method for timing fungicide sprays.

In Canada, Sutton *et al.* (1986) developed BOTCAST for the management of *B. squamosa* (onion leaf blight). They made the following assumptions on the epidemiology of *B. squamosa* (leaf blight) for developing BOTCAST, based on the literature:

1) Initial inoculum was always present,
2) Wet periods required for conidial production by sclerotia in crop residues or soil and by mycelium in dead leaves of the current crop were similar,
3) Temperatures during the wet period at night invariably favoured sporulation,

4) Conditions favourable for spore dispersal occurred each day, and
5) Portions of the spore population produced at night survived until wet periods of the first or second night after sporulation.

BOTCAST operated by following a set of rules to give an estimation of a daily inoculum value (DINOV) and a daily infection value (DINFV). From these values, a disease severity index was calculated for the day by multiplying the DINOV by the DINF value. The final step was the computation of the cumulative disease severity index (CDSI) by adding the daily DSI values from when the onions emerged. The steps for using BOTCAST are described as follows in the paper by Sutton *et al.* (1986):

Step 1) Estimation of the daily inoculum value (DINOV).

The DINOV was 1 or 0 when sporulation was predicted or not predicted, respectively. A DINOV of 0 implied that no inoculum was present, that infection need not be considered (Step 2), and that the disease severity index was 0 (Step 3). In predicting the DINOV, inhibitory or lethal effects of high air temperatures on the pathogen while in the colonisation phase, and the effects of weather on sporulation were considered according to the following criteria:

a) Mean hourly air temperatures of $\geq$ 30°C for $\geq$ 4 h on at least one of the preceding five days. When these conditions were satisfied, the DINOV = 0. When they were not satisfied, and temperatures were < 30°C or were $\geq$ 30°C for < 4h on each of the preceding five days, Criterion B was considered.
b) Wetness duration of < 5 h on the green leaves during the previous night. When this occurred, sporulation was not predicted, and the DINOV = 0. When the wet period was $\geq$ 5 h, Criterion C was considered.

c) Wetness duration of > 12 h on the green leaves during the previous night. Given this condition, sporulation was predicted, the DINOV = 1, and Step 2 was invoked. Otherwise criterion D was considered

d) Wetness duration of 5-12 h on the green leaves during the previous night. Under these conditions, the incidence of sporulation depended on the moisture status of the dead leaves at dew onset. When the preceding day was dry (RH < 70% for ≥ 6h) and the onions received no rain or overhead irrigation, sporulation was not predicted and the DINOV = 0. When the preceding day was more humid and/or the onions received rain or overhead irrigation between 08 00 and 03 00 h, the DINOV = 1.

Step 2) Estimation of the daily infection value (DINFV).

The DINFV for the first or second night after sporulation was 0, 1 or 2, depending on the temperature and duration of the wet period (Figure 1.5). Wet periods that exceeded 24 h were divided at 12 00 h when calculating the DINFV.

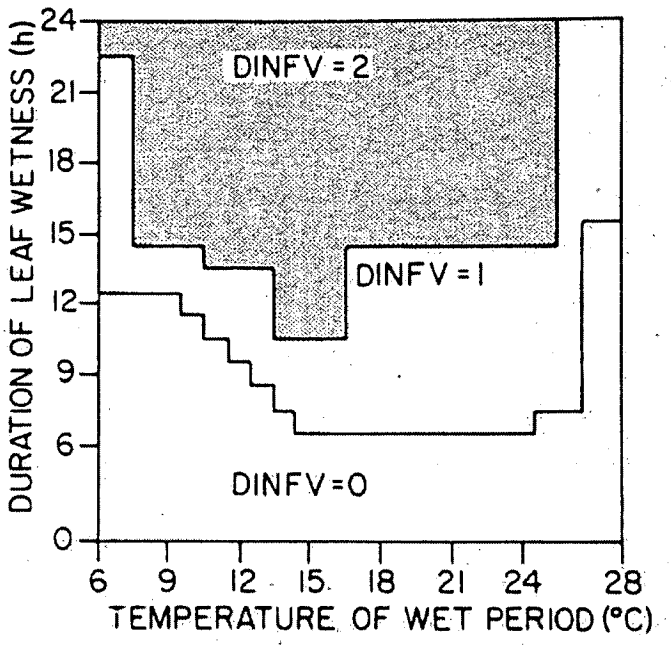

Figure 1.5: Chart for determining daily infection values (DINFV) for the disease prediction model BOTCAST based on the duration and mean hourly temperature of the leaf wetness period (Taken from Sutton *et al*, 1986).

Step 3) Computation of the disease severity index (DSI) for the day.

DSI = DINOV * DINFV

Step 4) Computation of the cumulative disease severity index (CDSI).

Daily DSI values were added from when the onions emerged, to calculate the CDSI.

BOTCAST was utilised to determine spray thresholds, measured as set values of CDSI. A CDSI between 21 and 30 was the first threshold and a CDSI value between 31 and 40 was the second threshold value. At threshold 1, the fungicide was not applied unless rain was forecasted or before overhead irrigation was applied, as there was only moderate risk of rapid disease increase. At threshold 2, the initial spray was applied as soon as possible as the risk of rapid disease incidence was considered high (Sutton *et al.*, 1986).

Using the thresholds of BOTCAST for timing the application of fungicides, Sutton *et al.* (1986) found that the crops required 1-2 and 2-6 fewer sprays using thresholds 1 and 2, respectively, for timing application.

BOLEB (*Botrytis* leaf blight prediction model) was developed by Alderman *et al.* (1987). The computer model simulated the build-up and spread of *Botrytis* leaf blight of onion, using inputs of daily averages of vapour pressure deficit and temperature. BOLEB was constructed with four basic components: (a) lesion production, (b) lesion expansion and extent of tissue blighted, (c) amount of spore release, and (d) fungicide spray options.

Vincelli and Lorbeer (1988ab) in New York State evaluated the sporulation index model (of Lacy and Pontius, 1983) and the DINOV submodel of BOTCAST (Sutton *et al.*, 1986). They found these models were sometimes poor predictors of spore release. They developed an alternative inoculum production index (IPI) model, using seven years of spore trap data and weather data.

Blight-Alert (Vincelli and Lorbeer, 1989), was developed for timing applications of protectant fungicides to control leaf blight, after it had reached the critical disease level of 1.0 lesion per leaf and after the first fungicide spray had been applied. Blight-Alert incorporated the inoculum production index (IPI) and National Weather Service forecasts of precipitation probability (PP). The BOTCAST and CDL models did not consider appropriate timings of fungicide applications after the first spray had been initiated. Use of either the 30-IPI treatment (PP $\geq$ 30% and an IPI forecasting

concurrent sporulation) or the 30-IPI2 treatment (where IPI2 is based on crop age not calendar days), for timing fungicide applications resulted in two to three less sprays than a weekly spray program, and controlled *B. squamosa* as effectively as the weekly spray program. The 30-IPI2 treatment was called BLIGHT-ALERT and was recommended by the authors to be used as a predictive system for forecasting infection periods of B. *squamosa*, thereby warning of periods when fungicide protection may be necessary, after the CDL had been reached.

BOTCAST and another model also based on historical weather data, SIV (for *B. squamosa*) were evaluated between 1988 and 1992 in the Netherlands (de Visser, 1996). The SIV model produces a Sporulation Index Value, which is based on hourly measurements of temperature and relative humidity. BOTCAST from Sutton *et al.* (1986) was used by de Visser (1996) to time the initial fungicide application, while the SIV model from Lacy and Pontius (1983) was used by de Visser (1996) for every subsequent fungicide application. Using both models in combination, de Visser (1996) reduced the weekly spray regime by 54%, without any negative crop health or yield effects.

Disease forecasting systems have been developed for *B. squamosa, B. cinerea* and many other fungal pathogens (de Visser, 1996: Shtienberg and Elad, 1997). A number of models have been produced for the forecasting of *B. cinerea* epidemics in vineyards and greenhouses. BOTMAN (short for *Botrytis* manager) was developed by Shtienberg and Elad (1997) for the integrated biological-chemical control of *B. cinerea* in greenhouses. Predicted weather information (provided by the Israel Weather Forecasting Service) proved to be more useful for disease warnings than

immediate past weather. However, forecasting systems have not been developed for *B. allii* due to the scarcity of information on the epidemiology and control of this pathogen. From the models that are available for related fungi, it may be possible to predict what parameters will affect development of *B. allii*. A review of the disease modelling literature may also provide information on a particular variable factor(s) that may be crucial in the development of a *B. allii* disease forecast model.

Chapter 2

2 Molecular characterisation of *Botrytis* spp. associated with onion in Australia

2.1 Introduction

Botrytis aclada (syn. *B. allii*) has previously been subdivided into two groups (AI and AII) based on spore size and chromosome number (Shirane *et al.*, 1989). Recent molecular studies by researchers in Denmark have also described two genetically distinct types of *Botrytis aclada. B. aclada* type AII (*B. allii*) was demonstrated to be a polyploid hybrid resulting from interspecific hybridisation of *B. aclada* type AI and *B. byssoidea* (Nielsen and Yohalem, 2001).

Nielsen *et al.* (2002) developed sequence-characterised amplified region primers (BA2f/BA1r) for the amplification of *Botrytis* spp. associated with neck rot of onions. Digestion of the polymerase chain reaction (PCR) amplification product from isolates of each species, using the restriction enzyme *Apo*I, made it possible to distinguish the five groups: *Botrytis aclada* types AI and AII, *B. byssoidea*, *B. squamosa*, and *B. cinerea*. A molecular survey of the *Botrytis* spp. associated with onion neck rot in Australia was conducted using the molecular techniques of Nielsen *et al.* (2002). Currently the *Botrytis* spp. associated with neck rot in Australia have been identified as *B. allii* on the basis of culture characteristics and spore size, with no published records of *B. byssoidea* or *B. squamosa* in Australia. Given the differences in spore size between *B. aclada* type AI and AII, it is possible that there are important biological differences between them in pathogenicity and epidemiology. This survey

was conducted to clarify the taxonomic position of *Botrytis* spp. associated with neck rot in Australia.

2.2 Materials and Methods

2.2.1 Collection of *Botrytis* isolates

Botrytis isolates were collected from bulb samples taken from the packing companies in the Forth region of Tasmania, from seed samples grown on mainland Australia and from isolate collections (Table 2.1).

Table 2.1: Origin of isolates of *Botrytis* spp. characterised by the SCAR primers and *Apo*I digestion, according to Nielsen *et al.* (2002).

Isolate	Source	Tissue of origin
C3[A]	Tasmania	Seed
C4	Tasmania	Bulb
C26	Scottsdale, Tasmania	Bulb
C30	Tasmania	Seed
DAR 41698[B]	New South Wales	-
DAR 33222	New South Wales	-
DAR 28780	New South Wales	-
DAR 40042	New South Wales	-
DAR 27004	New South Wales	-
DAR 33860	New South Wales	-
MKS-VE #1[C]	South Australia	Seed
MKS-VE #2	South Australia	Seed
MKS-SR#1	South Australia	Seed
MKS-SR#2	South Australia	Seed
MKS-VR#2	South Australia	Seed
HM360#1[D]	Cressy, Tasmania	Bulb
HM360#2	Cressy, Tasmania	Bulb
HM367 (*B. cinerea*)	Barrington, Tasmania	Bulb
FF131[E]	Barrington, Tasmania	Bulb
FF214	Longford, Tasmania	Bulb
FF219	Deloraine, Tasmania	Bulb
FF307	West Pine, Tasmania	Bulb
FF324	Boat Harbour, Tasmania	Bulb
FF44019[F]	Cowra, New South Wales	Bulb

[A] Author's isolate collection

[B] Isolates from Michael Priest' culture collection, NSW Agriculture, Forest road, Orange, New South Wales.

[C] Seed lots collected from Agronico Pty, Ltd, 175 Allport ST East, Leith, Tasmania.

[D] Onion bulbs collected from packing shed of Harvest Moon – Forth Farm Produce (HM), Forth, Tasmania.

[E] Onion bulb samples were collected from the Field Fresh Tasmania (FF) storage room, Forth, Tasmania. Two seed lots were used for all of the crops sown by FF, regular cream gold and early cream gold, grown in the Derwent valley and found to have no *B. allii* present in seed tests.

[F] Red onion seed for this bulb crop originated from Sunseeds, Oregon/Idaho (U.S.A.) cultivar Tango.

2.2.2 Fungal isolates

Fungal isolates (Table 2.1) were maintained on pectin agar (Appendix 12.5.1) (Metcalf and Dennis, 1998). Isolates were either collected from diseased bulbs or seed. Isolations from seed were performed by surface-sterilising the seed in 3% sodium hypochlorite for 1 minute and then rinsing the seed twice in sterile de-ionised water. The seeds were placed on pectin agar and incubated at 22°C for 7 days before identifying and subculturing *Botrytis* colonies. Bulb or leaf tissue samples were incubated in a plastic food container lined with a moist tissue for 1 week to promote *Botrytis* conidiophore production. Conidia were removed from conidiophores with a sterile needle and placed onto and maintained on pectin agar in petri plates under sterile conditions. Plates were incubated in the dark at 20°C until required.

2.2.3 DNA extraction from fungal mycelium

Isolates of *Botrytis* were grown in 10 ml of 2% liquid malt extract in high-sided petri plates on a rotary shaker at 40 rpm on the laboratory bench for 10 days. Mycelia (0.05 – 0.1 g) were transferred to a 2.5 ml eppendorf tube for DNA extraction using a modification of the CTAB method (Moller *et al.,* 1992). Mycelial samples were suspended in 500 µl of 2% CTAB buffer (Moller *et al.,* 1992) and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and homogenised using 500 mg of glass beads (0.5 mm diameter) in a bead beater (Mini-Beadbeater™, Biospec Products, Bartlesville, Oklahoma, U.S.A.) for 3 minutes at 5000 rpm, for 30 second intervals. Following homogenisation, samples were centrifuged at 15,000 rpm for 5 minutes and the aqueous layer transferred to a new tube. DNA was further extracted from the aqueous layer with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated using cold 95% ethanol and washed with 70% ethanol. DNA

was dissolved in 50 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C until processed further.

2.2.4 PCR Amplification

PCR amplification was performed in a total reaction volume of 20 μl: consisting of 1 μl DNA template, 2 μl PCR buffer (10X), 1.2 μl $MgCl_2$ (25 mM), 0.25 μl dNTP (10 mM), 1 μl primer BA2f (10 pmol/μl), 1 μl primer BA1r (10 pmol/μl), 0.2 μl Taq (5 units/μl) and 13.35 μl ddH_2O. Primer sequences described by Nielsen *et al.* (2002) are listed in Figure 2.1.

BA2f *Botrytis* amplification	GTGGGGGTAGGATGAGATGATG
BA1r *Botrytis* amplification	TGAGTGCTGGCGGAAACAAA

Figure 2.1: *Botrytis*-specific primers

A GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT) thermo-cycler was used to amplify the DNA from each isolate with primers BA2f and BA1r, with the following program: 94°C (3 min), 1 cycle; 94°C (30 s), 60°C (30 s), 72°C (1 min), 29 cycles; 72°C (3 min), 1 cycle; 4°C hold. The PCR product was separated in 2% agarose gels (ICN Biochemicals, OH) in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under UV light.

2.2.5 Restriction digest analysis

To differentiate among the *Botrytis* spp., the PCR amplicon from each isolate was digested with the restriction enzyme *ApoI*, as described by Nielsen *et al.* (2002)

(Table 2.2). The digestion was performed in a total volume of 10 μl made up of 2 μl of PCR amplicon, 1 μl 10X buffer, 0.1 μl BSA 100X (100 μg/μl), 0.25 μl *ApoI* (4 units/μl) and 6.75 μl ddH_2O. A GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT) thermo-cycler was used to incubate the digests at 60°C for 1 hour. The digestion products were separated by electrophoresis in a 1.5 % agarose gel, stained and visualised as described for PCR amplification.

Table 2.2: Diagnostic band(s) of *Botrytis Apo*I digest (Nielsen *et al.*, 2002)

Species	Diagnostic band(s) of restriction digested PCR amplicon
B. aclada type AI	413 bp
B. aclada type AII (B. allii)	413 + 298 bp
B. byssoidea	298 bp
B. cinerea	250 bp
B. squamosa	269 bp

2.3 Results

Amplification of the isolates with the *Botrytis*-specific PCR primers (Nielsen *et al.*. 2002) gave products approximately 413 base pairs in length (Figure 2.2). Digestion of these products with *Apo*1 clearly differentiated these isolates into the groups described by Nielsen *et al.*. 2002. The majority (22) of these isolates were *Botrytis aclada* AII (syn. *B. allii*), whilst there was only one *B. aclada* AI. A single, *B. cinerea* isolate was also included in this study (Table 2.1) and its identity confirmed by this technique (Figure 2.2 and Figure 2.3).

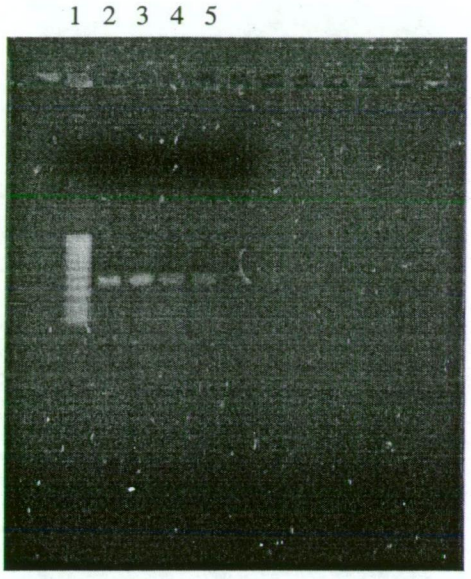

Figure 2.2: Lane 1 is a 100 bp ladder, lanes 2-5 are *Botrytis*-specific primer amplicons

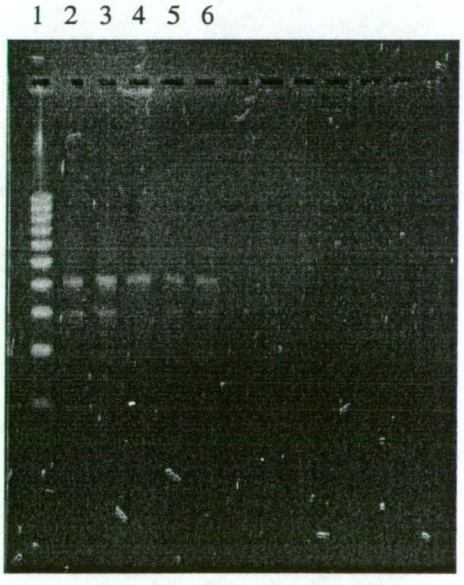

Figure 2.3: Lane 1 is a 100 bp ladder, lanes 2-6 are *ApoI* digests of the PCR amplicon of five *Botrytis* isolates, all indicating *B. aclada* type AII (*B. allii*). Lower band (298 bp) were observed in all five *Botrytis* isolates in gel.

2.4 Discussion

Botrytis aclada type AII (*B. allii*) was the dominant neck rot species of the isolates collected in this survey. By contrast, Nielsen *et al.* (2002) found approximately half of the *B. aclada* isolates included in that study were *B. aclada* type AI. Of 14 isolates they obtained from continental Europe, 8 isolates from bulbs of *A. cepa* and 1 from *A. schoenoprasum* were of type AI; and 3 isolates from bulbs of *A. cepa,* 1 from a leaf of *A. cepa* and 1 from *A. ascalonicum* were of type AII (Nielsen *et al.,* 2002). Of 6 isolates from the UK, 2 from bulbs of *A. cepa* and 1 from *A. ascalonicum* were of type AI; and 3 isolates from bulbs of *A. cepa* were of type AII. Of 5 isolates from the US/Canada, 2 isolates from bulbs of *A. cepa* were of type AI; and 1 from a bulb of *A. cepa,* 1 from unspecified tissue of *A. cepa* and another isolate of unknown origin were of type AII. Of 7 isolates from Japan, 3 from *A. cepa* were of type AI; and 3 from *A. cepa* and 1 from a leaf of *A. cepa* were of type AII. A single isolate from a bulb of *A. cepa* from Egypt was of type AII (Nielsen *et al.,* 2002).

The dominance of *B. aclada* type AII detected in onion seed and plant samples from Australia may possibly be related to differences in isolation frequency from onion tissues. *Botrytis aclada* type AI might be prevalent in Australia, but may be more difficult to culture than type AII thus being under-represented in this molecular survey. However, Nielsen *et al.* (2002) obtained a significant proportion of type AII isolates from culture collections, suggesting that type AII is easily obtained from onion tissue. Further possible reasons for the low proportion of type AI isolates in Australia may be that environmental conditions in Australia are less favourable for type AI isolates compared to AII isolates, or that an alternative preferred host of type AI isolates, such as an *Allium* weed species, may not be as prevalent in Australia as,

other countries, further experiments will be required to test these hypotheses. The differences in spore size between isolates types of AI and AII (Shirane, 1989) suggest that there may be other biological differences between the two types which may lead to difference in pathogenicity and epidemiology. Molecular methods for the detection and differentiation of neck rot species will enable studies to be undertaken to fully comprehend differences in disease epidemiology and ecological fitness among species of *Botrytis* pathogenic on onion.

No isolates of *B. squamosa, B. byssoidea* were found in the survey conducted. *B. cinerea* was routinely found and differentiated morphologically from the other *Botrytis* spp.

Chapter 3

3 Prevalence and incidence of *Botrytis allii* infection in Tasmanian onion crops

3.1 Introduction

A survey was conducted to assess the prevalence (the number of crops that are infected, out of the total number that are surveyed) and the incidence (the percentage of plants that were infected) of *B. allii* in Tasmanian onion crops during the 1999/2000 season. The secondary aims were to identify highly infected crops for spatial analysis of epidemics and to target highly infected crops for predictive bulb rot experiments (Chapter 4).

To the author's knowledge, field surveys of *B. allii* have not been published previously, either in Australia or other countries. This is probably due to the difficulty of detecting *B. allii* in the field. *Botrytis allii* is a perthophyte (although not always the case), i.e., it produces no structures or symptoms (e.g., leaf lesions) on healthy tissue and will only produce conidiophores once infected tissues senesce. This makes assessment of infection in the field difficult. Under ideal conditions, conidiophores of *B. allii* can be seen on senescing leaves or bulbs. However, differentiating between conidiophores of *B. allii* and *B. cinerea*, which is often also present on infected onion plants, can only be done with confidence using a dissecting microscope or by culturing the fungi on artificial media.

Currently, there exists no quantitative assay for determining *B. allii* infection in an onion crop, apart from collecting whole plants or parts of plants for incubation under

humid conditions, and subsequent observation of *B. allii* conidiophores. This is laborious, time consuming and not feasible on the scale that may be required for implementing strategic control measures. Only recently, has DNA based PCR detection of *Botrytis spp* in onions become available (Nielsen *et al.*, 2002). Other fungal pathogens like onion downy mildew (*Peronospora destructor),* which produce characteristic symptoms and signs of infection, can be scouted for in the field.

3.2 Methods

3.2.1 Crop selection for sampling

Early- and late-season onion crops of different maturities and at a range of geographic locations in Northern Tasmania (Figure 3.1, Figure 3.2, Table 3.1) were selected for the survey.

Table 3.1: Coordinates of sample sites, using UTM position format and Australian geodetic 84-map datum.

Site Label	*Easting and Northing*
A	55 G 404200 5452800
B	55 G 435000 5435000
C & D	55 G 439400 5439700
E	55 G 438900 5437700
F	55 G 438600 5438300
G	55 G 438800 5439300
H	55 G 433700 5436500
J	55 G 439900 5441500
K	55 G 437900 5437400
L	55 G 425700 5445600
M	55 G 427500 5444300
N	55 G 432500 5442400
O & P	55 G 543801 5447514
Q[1]	55 G 456500 5439500

[1]Map reference was not available so an approximation of location was made

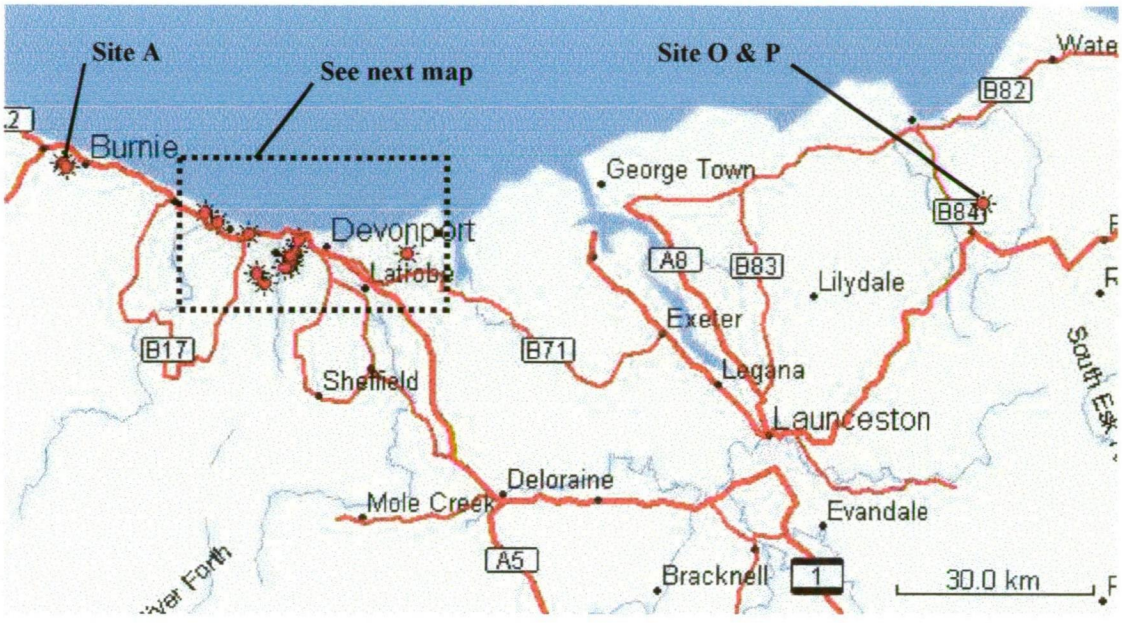

Figure 3.1: Map of onion crops selected for sampling across the north of Tasmania.

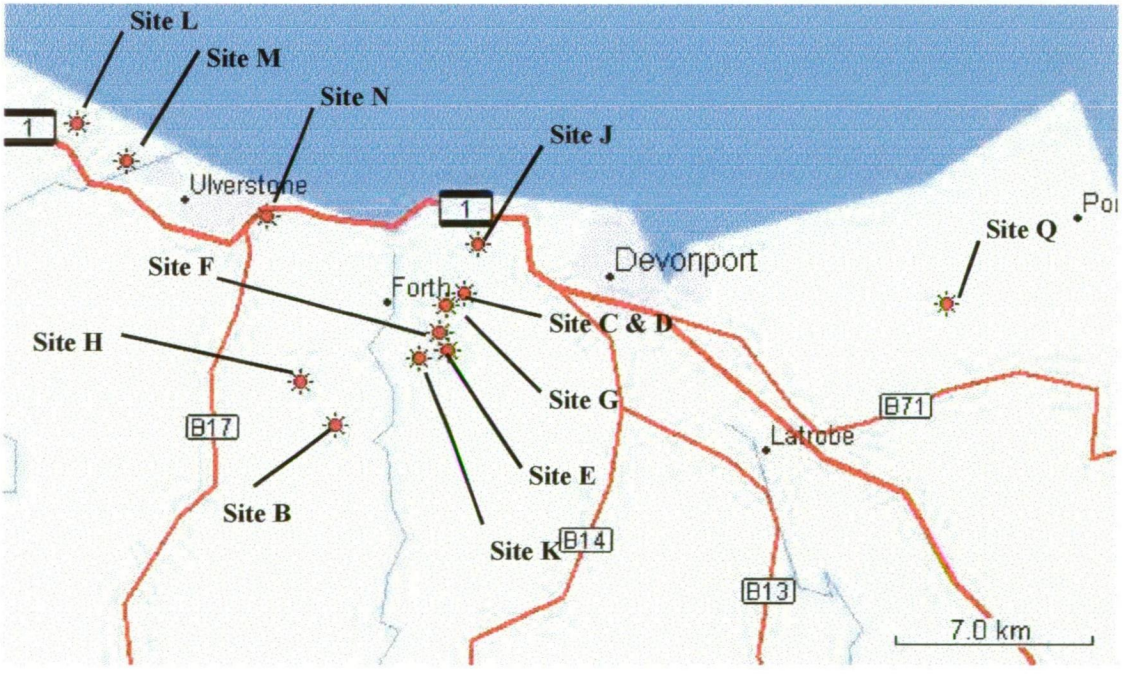

Figure 3.2: Map of sample sites in the major onion growing region, demonstrating the proximity of crops.

All onion crops sampled were of the variety 'Cream Gold', which is the main variety grown in Tasmania. Initial surveys were undertaken from late October to late December 1999. Additional samples were taken from three selected crops, C, D, and G, in January of 2000 (Table 3.2).

Commercial onion fields in Tasmania are usually from 2-10 ha up to a maximum of 20 ha in size and vary in topography and shape. The average size is usually around 6 ha (Dane Gilham, Field Fresh Tasmania, *personal communication*). The population of an onion crop is approximately 650,000 plants per ha (D. Gilham, *personal communication*). When sampling a large binomial population, sample size determines the accuracy of the population representation. Therefore, sample size of 1000 leaves was chosen. At the 99% confidence level, the incidence of infection would be no worse than 0.53% in the sample area if no infected leaves were found (Johnston and Kotz, 1969).

To maintain uniformity of the sampling and obtain a representative sample of plants per unit area, a 'W' pattern was chosen for sampling, covering an area of 0.8 ha. The 'W' pattern ensured a representative number of sample units were taken throughout the sample area and crop edges were not given priority but were included in the sampling area, enabling identification of any edge effects if they occurred (Barnett, 1986). The 'W' pattern was set up across 50 beds (approximately 80 m) of onions, and was 100 m in length. The 50 beds of onions were crossed four times, once on each leg (transect) of the 'W' sampling pattern. Each time a bed of onions was crossed, five leaves were randomly selected from across the bed following the line of the transect. Leaf samples were taken back to the laboratory for processing on the day

of collection. As the sampling area was smaller than the onion paddock, the chance of observing infection due to an edge effect was increased by locating the edge of the sample area on the crop edge. Where possible, the sample area was placed adjacent to the location of the previous year's crop, as this was hypothesised to be one of the most likely sources of inoculum of *B. allii* for the current season's crop. The 'W' pattern enabled samples to be taken from the same location at a later date.

The sample unit was the oldest (most basal) green leaf (excluding dead dry leaves) on the sample plant. This sample unit was selected because it was hypothesised that the older leaves were more likely to harbour infection by *B. allii,* similar to *B. squamosa* infection (Alderman and Lacy, 1984b). Furthermore, the oldest green leaf would have been exposed to potential infections for a longer period than younger leaves.

In the laboratory, the five leaves from each bed were placed into a non-sterile 1 L Genfac® plastic food tray (length 16 cm, breadth 10 cm and height 6 cm), lined with a moistened tissue and sealed with a lid. The trays were labelled according to transect and bed number so the spatial position of infected plants could be estimated. The trays were incubated at room temperature (15°C to 20°C) on the laboratory bench for 7 days to allow the leaves to senesce. Leaves were observed with bright light and a dissecting microscope (40 X magnification) for the presence of *Botrytis* conidiophores. The presence or absence of *B. allii* was recorded for each sample. As reported later, conidiophores of *B. allii* are much shorter and less branched than those of *B. cinerea.* To confirm identification, a total of 110 isolations were made from conidiophores to ensure correct visual identification and differentiation of *B. allii* from *B. cinerea.* Isolations were performed under a stereo microscope (40 X). Conidia

were removed from conidiophores with a sterile needle and placed onto Pectin agar (Appendix 12.5.1) in a petri plate under sterile conditions. Plates were incubated in the dark at 20°C. *Botrytis* isolates were identified (Hickman and Ashworth, 1943; Schwartz and Mohan, 1995) to species by their characteristic morphology on pectin agar (Appendix 12.7).

3.2.2 Onion bulb collection for correlation to leaf data

To compare the number of infected leaves with the incidence of bulb rot caused by *B. allii,* bulb samples (440 to 1122 bulbs) were collected from each of eight crops. The bulb samples were primarily collected from the factory after the crops were harvested. Bulbs were collected from bins and it was assumed that they formed a representative sample of the population from each field. Bulbs were stored for 4 months in 0.5 tonne wooden crates in an insulated shed. Ambient temperature in the shed varied from 10°C to 15°C and the RH varied between 65% and 98%.

Bulb samples were then cut in half longitudinally and the incidence of symptoms of neck rot and bacterial rot recorded. Neck rot was differentiated from other rots by the characteristic slightly water-soaked appearance (Figure 1.1, Section 1.2.2), and the presence of *B. allii* conidiophores and/or sclerotia.

3.2.3 Weather data

Weather data was obtained from the Forthside weather station in Forth (Figure 3.2), for the seasons 1995/1996, 1999/2000 and the average from 1990 to 2002, for comparison among seasons. Weather data for the 1995/1996 season was included as

this was a season where very high levels of neck rot occurred, due predominantly to wet weather conditions late in the crop life and during the harvest period.

3.3 Results

Botrytis allii was detected in leaf samples collected from 6 of the 16 crops surveyed from October to December of 1999. The incidence of *B. allii* ranged from 0.1% to 0.3%. In the three crops that were re-sampled in January 2000, incidence increased from 0.1% to 3.2% in crop C, 0.0% to 0.5% in crop D and 0.3% to 5.8% in crop G (Table 3.2). At the second sampling dates, crop C was at the 70% "tops down" stage, i.e., approximately 70% of the onion plants had leaves beginning to fall over as a result of maturity. The recommended harvest index is when 70% of the crop is at the "tops down" stage. Crop D had no plants "tops down", but was only approximately two weeks from commencement of this stage. Crop G was past 70% "tops down" and was due for lifting.

In Crop O at Scottsdale, bulbs were found with mycelium and conidiophores characteristic of infection by *B. allii*, present just below the soil line around the entire base (Figure 3.3). Isolations onto pectin agar confirmed the presence of *B. allii.*

Figure 3.3: Conidiophores of *B. allii* indicated with arrow, below the soil line on a bulb from Crop O.

Botrytis neck rot was found in the bulbs from all 8 crops sampled (Table 3.2). The incidence of neck rot ranged from 0.4% up to 16.3%. The average incidence of bulb rot amongst the commercial crops was 5.3%.

Botrytis allii bulb infection was differentiated from other bulb rots by the characteristic symptoms and signs of the disease. Neck rot generally induces a water-soaked lesion, mycelia and conidiophores of *B. allii* are often present and sclerotia can also be present. In contrast, most bacterial rots demonstrate a water soaked lesion and give off a foul odour. Positive identification of the casual agent was not always possible, as the pathogen that initiated the rot may have been overrun by secondary pathogens. In some very rotten bulbs, sclerotia similar to those of *B. allii* were found, confirming that rot most likely occurred as a result of *B. allii* infection.

Table 3.2: Incidence of *B. allii* in leaf and bulb samples from commercial onion crops in Tasmania during the 1999/2000 season.

Crop	Sowing date	Crop sample date	Crop age (days)	Approximate growth-stage of crop	Leaf sample size	% leaves infected with *B. allii*	% *B. allii* in bulbs (and bulb sample size)
A	6/8/99	25/10/99	80	2-3 leaf	880	0.0	0.4 (524)
B	15/7/99	28/10/99	105	3 leaf	1000	0.0	-
C time 1	2/7/99	2/11/99	123	4 leaf	1000	0.1	2.9 (547)
C time 2	2/7/99	18/1/00	200	70% tops down	1000	3.2	" "
D time 1	9/7/99	2/11/99	116	4 leaf	1000	0.0	2.6 (537)
D time 2	9/7/99	18/1/00	193	start of tops down	1000	0.5	" "
E	17/8/99	3/11/99	78	3-4 leaf	1000	0.1	4.8 (440)
F	2/7/99	3/11/99	124	4 leaf	1000	0.0	2.5 (636)
G time 1	3/6/99	3/11/99	153	4-5 leaf	1000	0.3	15.6 (900)
G time 2	3/6/99	28/1/00	239	>70% tops down	1000	5.8	" "
H	-	5/11/99	-	3-4 leaf stage	1000	0.0	-
J	4/6/99	17/11/99	166	bulbing	1000	0.2	-
K	5/8/99	19/11/99	106	bulbing	970	0.0	-
L	1/6/99	19/11/99	171	bulbing	1000	0.0	8.5 (1122)
M	5/6/99	24/11/99	172	bulbing	1000	0.3	-
N	5/8/99	24/11/99	111	bulbing	1000	0.0	-
O	24/4/99	2/12/99	222	bulbing	1000	0.2	16.3 (1132)
P	27/7/99	2/12/99	128	bulbing	1000	0.0	-
Q	-	22/12/99	-	1-week from lifting	1000	0.0	-

The correlation coefficient between the first leaf samples and the level of bulb rot was $r = 0.83$, significant at P=0.05. A linear regression analysis indicated an R^2 value of 0.63 for the regression equation $y = 45.3x + 2.74$, where y = the neck rot % found in bulbs and x = the *B. allii* % found in the early-season leaf sample. However, it should be noted that the relationship was only based upon a sample of eight data points.

Isolations onto Pectin agar were performed for various fungal cultures resembling *Botrytis* for each of the crops surveyed to determine which species was present. However, under uniform laboratory conditions of leaf senescence, *B. allii* and *B. cinerea* conidiophores could be differentiated by eye reliably (Figure 3.4). *Botrytis allii* produces short (usually about 1 mm tall) conidiophores with occasional branches. Conidia can also be clustered up the conidiophore stalk. *Botrytis cinerea* produces much longer (> 2 mm) conidiophores with long branches.

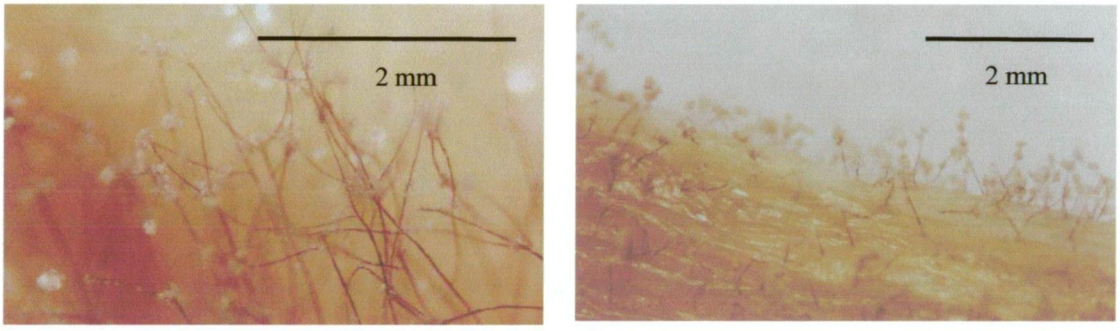

Figure 3.4: Conidiophores of *B. cinerea* (left – 18X magnification) and *B. allii* (right - 10X magnification).

In the field, various environmental and host factors make it very difficult to differentiate *B. allii* and *B. cinerea* visually. For example, under dry conditions, *B. cinerea* may produce shorter conidiophores similar to *B. allii.* Although caution must be exercised even in the laboratory, it was deemed unnecessary to subculture from every *Botrytis* conidiophore observed on the samples. The method of visually separating *B. allii* and *B. cinerea* on incubated leaf samples was also validated in the field trial experiments (Chapter 7).

Figure 3.5: Rainfall recorded at Forthside weather station, for the 95/96 (—) and 99/00 (—) seasons and the average of 1990 to 2002 (–).

The 1999/2000 season had close to average rainfall during the months of January and February 2000 (Figure 3.5), the months during which onion bulbs are cured in the field, and probably the most critical in determining quality and level of neck rot in bulbs. The 1995/1996 season was included in Figure 3.5 for comparison, as during this season very high levels of neck rot were recorded, due mainly to the very wet period late in the growing season and during the field curing period.

3.4 Discussion

Botrytis allii was prevalent in commercial Tasmanian onion crops during the 1999/2000 growing season. It was identified in foliar samples from 6 of 16 crops sampled and in bulbs from all 16 crops sampled. The incidence of *B. allii* in leaf

samples was found to be low early in the season (pre-bulb initiation) and mid-season (during bulbing).

Results indicated that the incidence of *B. allii* is very low early in the season. For example, even at a sampling intensity of 1000 leaves/ 0.8 ha, *B. allii* was not detected in Crop L. However, the final level of neck rot in the harvested and stored bulbs was 8.5%. However, caution is required as the area in the field from which leaf and bulb samples were taken was not the same. The incidence of bulb rot in crop L was above the commercially-acceptable level of bulb rot (less than 5% total rots, Carey, *personal communication*). For the 3 crops from which leaf samples were collected a second time (approximately 2.5 months later), the incidence of infection had increased considerably for the 3 crops, and the incidence noted was a more accurate reflection of the incidence if neck rot that developed. It is possible in 2 of the 3 crops where the level of neck rot was higher than the level of leaf infection may have occurred due to the time between leaf sample and harvest interval, where infection may have spread among the onion plants. The conditions during curing may also have resulted in differences between *B. allii* leaf infection incidence and the incidence of neck rot, as wetter conditions during curing are more favourable to leaf infection moving into the neck of the onion bulb than dry conditions. During the 1995/1996 season, wet conditions at the time of curing led to significant losses in the Tasmanian onion industry due to rots caused, in particular, by *B. allii* (Dennis, 1996). Given the prevalence of *B. allii* detected in crops during 1999/2000, it is possible that disease incidence in bulbs would have been much higher had higher levels of rainfall occurred this season. For example, wet conditions over the growing season would allow for more secondary spread within the crop, and wet conditions at lifting with slow leaf

drying would allow *B. allii* greater opportunity to move from infected leaves into the necks of bulbs as the leaves senesce.

Maude and Presley (1977a) found that once leaf tips had been infected by *B. allii,* the fungus would move down the leaf and enter the neck of the bulb. If bulbs were not infected before lifting, a sufficiently quick curing process could prevent infections residing in leaves from entering the necks of the bulbs.

A crop sampled at Scottsdale (Crop O) had unusual disease signs not noted in any of the other crops that were surveyed. *Botrytis allii* conidiophores were found below the soil line on green onion bulbs. Infected bulbs appeared to be spread throughout the crop, representing approximately 10% of the bulbs. However, the bulbs with the *B. allii* conidiophores did not have any rot symptoms. Identification of the *B. allii* conidiophores was confirmed by isolation onto pectin agar. A sample of 1000 leaves taken from 0.8 ha of the crop area detected *B. allii* in only 0.2% of the leaves while neck rot of bulbs was 16.3%. Furthermore, the location of rot was not noted to be unusual i.e., no high proportion of rot located around the base of the bulb. The absence of high levels of detectable leaf infection at this site may have resulted from applications of Benlate® (benomyl). The last application was made only 7 days prior to leaf sample collection and may have eradicated the fungus from the leaf or prevented it from growing out during incubation. The application of Benlate sprays may not have reached the fungi on the basal part of the bulb. If the leaf/scale that the fungus is living on is no longer receiving nutrients and water from the bulb, then fungicides will not be translocated to the tissue supporting the fungus. As Crop O was infected with *B. allii,* a check was made of the neighbouring crop (P), located

approximately 50 m away, established with a different seed line. No infection by *B. allii* was found in Crop P. The Crop O site had not had onions grown in it for the previous 4 years, which is the industry standard to reduce the likelihood of *B. allii* sclerotia or *B. allii* infected soil or debris from infecting subsequent crops. The source of infection may have been the seed-line that was used to establish the crop. However, a seed sample was not available to assay for this crop.

Basal infection, as observed in Crop O, may have occurred as a result of the fungus growing down the senescing leaf and reaching the base of the bulb. Alternatively, spores may have washed from infected leaves down into the soil where they infected the base or roots of the onion plant. Infection of the base may also result from *B. allii* residing in the soil from previously infected crops in the form of sclerotia, or perhaps mycelia. Extreme fluctuations in environmental conditions may be avoided below the soil-line, as soil is usually moist in this zone, thus providing suitable conditions for sporulation of *B. allii.* In Turkey, Ozer and Omeroglu (1995) isolated a higher rate of *B. allii* from onion roots (23.8%) than onion bulbs (10.1%). It was also found that *B. allii* was capable of infecting unwounded onions when placed into soil in contact with the plant (Ozer and Omeroglu, 1995). Anecdotal evidence from industry (D. Metcalf and J. Dennis, *personal communication*) suggests that basal rot is a significant form of loss associated with *B. allii* in Tasmania. Further work is required to examine this phenomenon.

Chapter 4

4 Predicting the level of latent neck rot in onion crops

4.1 Introduction

Background

Symptoms of *B. allii* infection are rare on foliage of onion, so crops cannot be visually assessed to determine the level of disease. Similarly, infections in the bulb are often initially latent and rots do not show up for some weeks in storage. As a result, infected bulbs cannot be detected at grading. The inability to detect infected crops is of concern to onion exporting companies, as crops which appear perfect when packed, may rot in transit and storage.

A method to assess the incidence of infection in crops prior to shipment would be of benefit to the industry, to ensure a constant supply of quality bulbs to market. Detecting diseased bulbs prior to a storage period has been attempted in the past, using three main methods, 1) Kritzman's stain, 2) an immuno-assay and 3) by conventional isolation. Recently, a molecular technique was developed for the detection of *B. allii* (Nielsen *et al.*, 1999 and 2002).

Kritzman's stain

Kritzman's stain (Kritzman, 1983) is based on a dye which changes colour with a change in pH. *Botrytis* produces oxalic acid as it grows, thereby lowering the tissue pH. Infection is detected by spraying Kritzman's stain onto cut bulbs and observing a colour change as a result of infection. Kritzman (1983) used an incubation period to

encourage *B. allii* from the latent phase into an active phase for detection. Incubation was conducted by placing bulbs into cool storage (4°C) for 2 days and then at 15°C for 16 days prior to cutting the bulbs in half and spraying the cut surfaces with the stain. Kritzman (1983) achieved the greatest detection of infected bulbs by incubating the bulbs for a further 14 days prior to staining. However, a time period of 32 days incubation to determine the level of disease in a crop is of little value to an industry where results are needed shortly after harvest (lifting), or earlier. Kritzman's stain was assessed in Tasmania (Metcalf and Dennis, 1998), but it was concluded that a more rapid method of detection was required, as infection of bulbs was not detected until the *Botrytis* infection began to develop into symptomatic neck rot. Metcalf and Dennis (1998) also raised concern that other factors may influence the pH of bulb tissue and result in an incorrect diagnosis.

Immunological detection

Immunoassays have been widely utilised in plant pathology for detection of pathogens. In the United Kingdom, a polyclonal antibody (Pab) was developed against *B. allii* by Linfield *et al.* (1995). A potential problem with using polyclonal antibodies for detection of fungi is lack of specificity, leading to cross-reaction with other (often related) fungal species. The Pab developed by Linfield *et al.* (1995) from cell wall extracts had only minor cross-reactivity with *B. cinerea.*

Linfield *et al.* (1995) indicated the progression of *B. allii* infection around the neck of the bulb was not uniform in the early stages of infection. A higher incidence of infection was detected soon after harvest (lifting) when testing neck tissue by an enzyme linked immunosorbent assay (ELISA) than by conventional isolation. However, at the end of a storage period of 131 days, the results of conventional

cultural techniques of isolating for *B. allii* were similar to the results when testing by ELISA. Cultural techniques rely on propagules of the fungus being present in the tissue sample cultured. The increased sensitivity of the ELISA at the earlier sampling period, compared to conventional isolation, suggested that *B. allii* infection could be detected in samples not containing mycelia or spores by detecting antigenic substances produces either as metabolites or following liberation of cell contents after membrane lysis (Linfield *et al.*, 1995).

An attempt was made to raise polyclonal antibodies against *B. allii* at the University of Tasmania (Mackie, 1997). However, the antiserum was unable to distinguish between *B. allii* and *B. cinerea.*

Increased specificity of an ELISA could be obtained by producing a monoclonal antibody to *B. allii.* However, this technology requires specialised and expensive laboratory facilities which were not available during this study.

Incubation of onion tissue

The traditional technique to assess latent *B. allii* infection is to incubate onion tissue pieces in a humid environment. Incubation induces plant tissue to senesce and promotes sporulation and disease expression. Metcalf and Dennis (1998) cut the tops off onion bulbs and separated the scales to determine where most infections of *B. allii* occur. Of 22 infected bulbs, infected tissue was found in the fleshy scales just under the outer dry scales in 21 bulbs and 13 of the 22 bulbs also had infections in deeper (Metcalf and Dennis, 1998).

In a similar study in Holland, parts of the neck region (inner leaf blades) were placed onto water agar, incubated and sporulation observed (Chris de Visser, *personal communication*). The relationship between percentage of bulbs with neck rot and *Botrytis* detected in the neck-pieces was:

Neck rot (%) = 7.1 +1.4 X percentage *Botrytis*-infected in neck pieces, where bulbs were field cured.

In two other trials by de Visser, the following relationship was found, where bulbs were cured artificially cured:

Neck rot (%) = 4.5 + 1.34 X percentage *Botrytis* found in neck pieces

Neck rot (%) = 18.6 + 1.34 X percentage *Botrytis* found in neck pieces

Experiments were conducted in Tasmania (Jason Dennis, Field Fresh Tasmania, *personal communication*) to determine the most suitable section of a bulb to sample for a routine test for bulb infection by *B. allii*. Onion tops were cut from bulbs using a knife, or an apple corer was used to remove a core from the neck of an onion bulb or to remove a core from the base of a bulb. Up to 8 pieces of onion (i.e. top section or neck core or base core) were incubated in a Genfac® food tray for 7 days and assessed for infection. A comparison of the results of incubating 49 onion bulb tops, 46 cores from the top of the neck of the bulb or 39 cores from the bulb base, led to an incidence of detected infection of 14%, 13% and 3%, respectively.

Molecular techniques

Nielsen *et al.* (2002) recently developed two sequence characterised amplified region (SCAR) primers (BA2f/BA1r) that amplify a 413-bp PCR product in the five *Botrytis* groups associated with onion diseases. The PCR primers exclusively detect *Botrytis* spp. and an *ApoI* digest can be used to distinguish between the following 5 *Botrytis*

spp.: *B. aclada* types AI and AII (*B. allii*), *B. byssoidea, B. squamosa* and *B. cinerea.* The detection limit for the primer pair developed by Nielsen *et al.* (2002) was between 1 and 10 pg of pure fungal DNA. This method detected *B. aclada* in onion leaf tissue 48 hours after inoculation with conidia, when no visible symptoms were present. Latent infections were detected in three of 60 onion leaf pieces using the PCR method, where the standard isolation procedure only detected infection in one of the 60 leaf pieces (Nielsen *et al.*, 2002). The technique was also used to detect *B. allii* latent infections in bulb tissue (Nielsen *et al.*, 1999). Using the PCR-method, a two- to three-fold higher detection rate was found compared with conventional culturing techniques, from a sample of 320 onions (Nielsen *et al.*, 2002). The method developed by Nielsen *et al.* (2002) may be of use in detecting and identifying latent infections of onion bulbs by *Botrytis* species and may have potential as a seed testing.

Objectives

Experiments were carried out in 1999 and 2000 to develop further a tissue incubation technique as a tool for predicting the amount of neck rot that would develop in onion bulbs in storage. Such a technique would be useful for onion companies in making decisions regarding onion bulb storage, handling, marketing and export.

To further improve the accuracy of prediction, trials in 2001 were conducted under more controlled conditions for coring onion bulbs, i.e., in a laminar flowhood. A modified coring procedure was also tested to determine if it could predict more accurately the level of neck rot that developed in storage. The effect of the time of coring after lifting was also investigated further.

4.2 Materials and methods

Definition of seasons

Within this chapter, data was collected over three onion growing seasons. The seasons run from May, when sowing begins, through to February the following year, when the majority of crops are harvested. Data was collected during the 1998/1999, 1999/2000 and 2000/2001 seasons which, for brevity, will be referred to as the 1999, 2000 and 2001 seasons, respectively.

4.2.1 1999 season

The population of an onion crop is approximately 650,000 plants per ha (Dane Gilham, *personal communication*). Therefore, to detect individual diseased bulbs, sample size and sampling procedure is of great importance. When sampling a large binomial population, sample size determines the accuracy of detection. If 550 bulbs are sampled from an infinite population and no infected bulbs are found, the disease incidence can be no worse than 1% for a confidence interval of 99% (Johnston and Kotz, 1969). A sample size of approximately 550 bulbs was used in the 1999 and 2000 season to detect any crops with low levels of infection by *B. allii.*

Bulb samples from 10 onion crops and 1 crop of shallots in northern Tasmania were collected and processed at Field Fresh Tasmania, Devonport (Table 4.2). For each crop samples in 1999, neck cores from 360 to 580 bulbs per crop were assessed for *B. allii* infection as described below. A further 413 to 636 bulbs per crop were stored at ambient temperature and humidity inside a shed and were assessed for bulb rot approximately 4 months after lifting. For the 1999 season, bulbs were collected from the factory after harvest.

The relationship between incidence of *B. allii* in neck cores and bulbs was examined by determining the coefficient of correlation (r) and comparing this with statistical tables of significance for r (Steele and Torrie, 1960). Significant correlations were further examined by regression analysis.

The crops labelled 225 April and 225 May (Table 4.2) were from the same location (and thus labelled as the same crop by the onion company) but were separate fields sown in April 1998 and May 1998, respectively. When these two crops were sampled, the bulb samples from each crop were divided into two groups for different treatments. The two sets of bulbs from Crop 225 April were used to examine the effect of an extra 2 weeks delay from lifting to coring on the ability to detect *B. allii* in neck core samples. The two samples from Crop 225 May were used to examine the effect of incubation period of the cores on the incidence of *B. allii* detected in the cores. One sample was assessed after 1 week of incubation (same incubation period used for all the other crop samples) and the other incubated for 2 weeks after coring (extended incubation period).

Bulb samples were collected from Crop 342 (Table 4.2) using two methods. The sample labelled '342 trial site' was taken from the location in the paddock where a *Botrytis* disease trial had been located 2 seasons earlier. During this trial, approximately 55% of the bulbs in the trial developed neck rot after a 4-month storage period. The cleanup of bulbs from the paddock was reported to be thorough, with the removal of all bulbs (Dean Metcalf, *personal communication*). An increased sample size (1282 bulbs) was taken from the area of the old trial site. The other sample from Crop 342 was collected from the factory and was derived from the entire crop area.

The protocol for onion bulb coring was as follows:

1. The tops of the onions were removed using secateurs if they had not already been topped in the field or factory.
2. An apple corer was used to remove a 2-cm-diameter by 3-4-cm-deep core from the top of the neck into the centre of the onion bulb.
3. The core was removed using a plastic rod and placed into a food tray (500 ml Genfac® take-away tray) lined with a moistened tissue. Eight cores were placed in each tray and the tray was sealed with a lid.
4. To prevent cross contamination, corers and rods were sterilised in 50% bleach (White King, 4.0% sodium hypochlorite) for at least 1 minute and given 2 rinses of distilled water between removing each core (several corers and rods were used to ensure adequate time for sterilising equipment).
5. Trays were incubated for one week on the laboratory bench (~20°C).
6. The cores were assessed under a bright light for the presence of fungal mycelium and conidiophores. Where necessary, a dissecting microscope was used for differentiation of *B. allii* from other fungi.
7. To ensure correct differentiation of *B. allii* from *B. cinerea,* isolations were performed on some of the cores that had *Botrytis* growth. Spores were picked off from conidiophores under a stereomicroscope (X40) and under aseptic conditions, plated onto pectin agar (Appendix 12.5.1) in a petri plate and incubated at 20° C. Pectin agar was used, as it was easy to differentiate *B. allii* and *B. cinerea* colonies on this medium based on colony morphology. See Appendix 12.7 for photos and details of the methods.

4.2.2 2000 season

In the 2000 season, bulbs were collected from 14 onion crops in northern Tasmania. Bulbs were collected in the field, to minimise the time between lifting and coring. Six of these crops had been assessed for leaf infection during the season (Chapter 3). Other crops were selected on the advice of field officers as being suspected of being infected with *B. allii*. During the 2000 season, samples were processed at the Tasmanian Institute of Agricultural Research (TIAR) laboratories, University of Tasmania, North West Centre, Burnie.

All bulb storage samples were assessed for *Botrytis* bulb rot at 16 weeks from the lifting date. The bulbs sampled from all crops from the 2000 season, except three (Field trial Crop, 404A Crop and 404B Crop), were randomised after sample collection, by removing all of the bulbs from the collection bags and randomly redistributing the bulbs into sub-samples.

In the 2000 season, bulbs were cored 2-7 weeks from the time of lifting. Bulb samples from five crops were cored two or three times, at 4 week intervals, to assess the effect of time of coring on prediction of neck rot. One sub-sample from each of these 5 crops (was placed into storage to determine the actual level of disease that developed, while a sub-sample was cored immediately.

Protocols for onion coring in 2000 were as described for 1999.

4.2.3 2001 season

Experiments in 2001 were conducted on bulbs from a single site under more controlled conditions than in 1999 and 2000. Diseased bulbs were obtained from a field trial crop (Field trial 2, Chapter 8, Section 8.2.3).

All coring was performed in a laminar flow cabinet to minimise risk of cross contamination. The corers (apple corers) and rods used to remove cores from the corers were surface-sterilised with 5% NaOCl, for at least 1 minute, and rinsed in changes of distilled water between bulbs. Fresh bleach solution was used between samples.

Experiments during 2001 investigated different coring procedures and the effect of time from lifting to coring on detection of *B. allii.*

4.2.3.1 Coring procedure (neck core and entire core as predictors) - 2001 season

Two coring methods were evaluated during 2001. The neck core was either performed as in previous seasons, or a core was taken which extended all the way though the onion, (entire core) including the basal plate.

Bulbs for the coring procedure experiments were collected from a field trial (Chapter 7) from a treatment area receiving a high rate of irrigation. Bulbs were collected from 5 beds (Beds 1,2,3,4 and 10) with different incidences of leaf infection (80%, 22%, 4%, 4% and 0%, respectively) to provide a range of bulb infection levels (Field trial 2, Chapter 8, Section 8.2.3). Samples from each bed were randomised after sample collection before being split into twelve sub-samples with 4 replicates of the following treatments: a neck core, an entire core and a storage sample. The storage

sample was stored in ½-tonne wooden potato crates, in an insulated potato shed at ambient temperature and RH.

The relationship between the incidence of *B. allii* detected in different core types and neck rot that developed in storage was analysed by linear regression using Genstat® Version 4.2.

4.2.3.2 The effect of time of coring after lifting on prediction of storage rot - 2001 season

The effect of time from lifting to coring on the relationship between predicted and actual neck rot was investigated. The experiment consisted of three times of coring after lifting. Bulbs were taken from 2 beds in a field trial from in a treatment area receiving a low irrigation treatment (Field trial 2, Chapter 8, Section 8.2.3). The two beds had 67% and 13% incidence of *B. allii* in the leaves. Four replicates of 40 bulbs were used for each treatment combination. On three separate dates (Table 4.1) the onion bulbs were cored (neck core only) to determine the effect of time from lifting to coring on the precision and accuracy of the coring test at predicting neck rot in storage. From each of the two beds infected with *B. allii*, 4 replicate samples of 40 bulbs were stored for 4 months then assessed for neck rot, as previously described.

Table 4.1: Sequence of events for the 2001 coring experiments

Date	*Days*	*Event*
5/03/2001	0	Crop lifted
28/03/2001	23	Bulbs collected from field for randomisation
9/04/2001	35	1st coring in time series and test of coring procedure experiment (neck and entire cores)
7/05/2001	63	2nd coring in time series
4/06/2001	91	3rd coring in time series
3/07/2001	120	Cut storage sample of bulbs to determine actual incidence of neck rot

4.3 Results

4.3.1 1999

Eleven crops of onions and one crop of shallots were cored during 1999 and the stored bulbs assessed for neck rot after a further storage period of 3-4 months (Table 4.2). *Botrytis allii* was detected in the cores taken from bulb samples of 10 of the 11 crops and in the stored bulbs of 9 of the 11 crops (Table 4.2). Replicate samples taken at the same time from each of Crops 225 (May) and 340 gave similar incidences of *B. allii* in the neck cores within each crop (Table 4.2). Neck cores taken from Crop 225 (April) at 11 and 13 weeks predicted a higher and lower (2.4% and 0.3%) incidence of neck rot, respectively, than the incidence in the stored bulbs (1.1%) (Table 4.2).

Table 4.2: *Botrytis allii* detected from core samples and in bulbs of onions and shallots after storage (1999).

Crop	*Coring assessment*			*Stored bulb assessment*		
	Weeks from lifting to coring	No. of cores	% *Botrytis* detected	Weeks from lifting to cutting	No. of bulbs	% *Botrytis* rot
104	19	544	9.6	29	539	13.2
209	19	541	0.9	28	552	2.4
225 (sown in April 1998)	11[b]	285	2.4	-	-	-
	13[b]	293	0.3	-	-	-
	Subtotal	578	1.4 (mean)	24	562	1.1
225 (sown in May 1998)	14	279	0.7	-	-	-
	14[a]	278	0.7	-	-	-
	Subtotal	557	0.7 (mean)	24	543	0.5
326	15	497	1.0	25	597	1.8
332	13	553	1.6	27	543	3.5
334	13	586	1.5	27	512	1.6
340	13	275	0.4	-	-	-
	13[a]	276	0.4	-	-	-
	Subtotal	566	0.3 (mean)	22	519	2.1
342	9	541	0.4	22	549	0.2
342 trial site	8	669	0.1	22	613	0.5
SG	19	501	0.0	30	622	0.0
Shallots	12	694	0.1	21	651	0.0

[a] Cores incubated in trays for 2 weeks instead of the standard 1 week used for samples from the other crops.

[b] One sample of crop 225 (sown in April 1998) was cored 11 weeks after lifting and another sample was cored 13 weeks after lifting. Each sample was collected from the field, whereas other crop samples were collected from the factories after lifting.

- The bulb samples cut for assessment were not split, thus only a subtotal value is expressed.

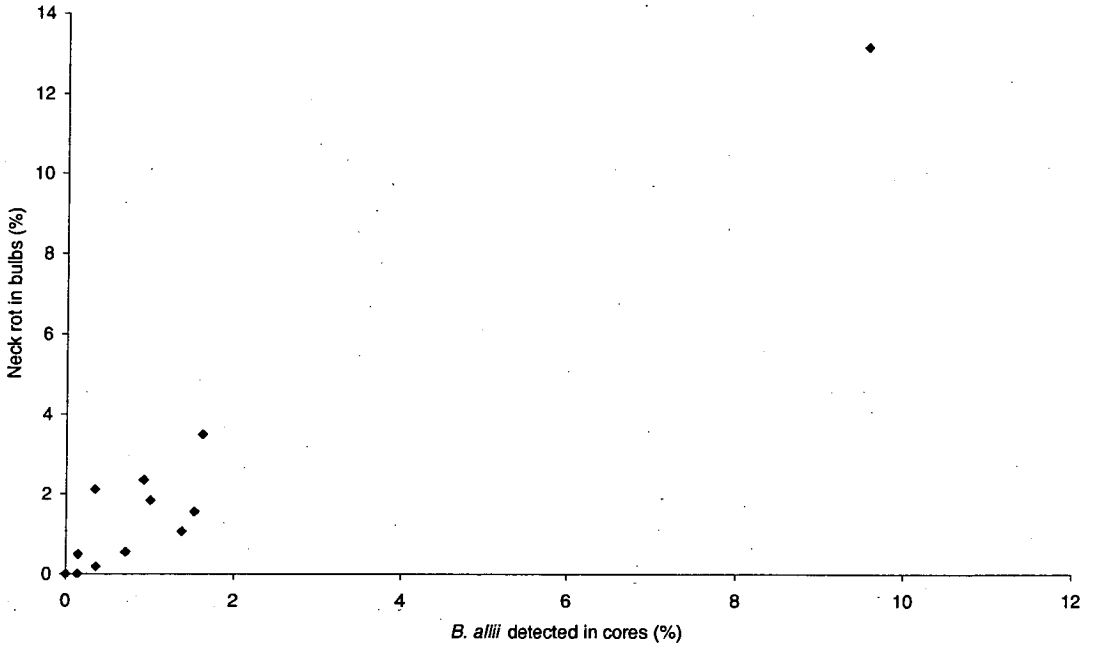

Figure 4.1: Incidence of *B. allii* detected in core samples and incidence of bulb rot in storage for the 1999 season.

The data for the 1999 season, did not provide an adequate distribution in incidence of *B. allii* detected in cores or in stored bulbs to make confident interpretations about the relationship between the predicted and actual level of *Botrytis* rot present in onion bulbs (all data points were below 2.0% and 3.5%, respectively). A single data point occurred at 9.6% predicted and 13.2% actual rot (Crop 104), which resulted in significant weighting in the regression analysis (Figure 4.1). Analysis of the whole 1999 data set gave a significant correlation and a high R^2 value (Table 4.3). The relationship was approximately 1 predicted bulb with neck rot to 1.4 actual bulbs with neck rot. This was similar to the relationship derived in a study in the Netherlands (Chris de Visser, *personal communication*).

Table 4.3: Correlation coefficients and regression analysis of the relationship between *B. allii* detected in cores and neck rot in bulbs (1999).

	Correlation coefficient and statistical significance	*Regression analysis*		
		Fitted linear equation	*F-probability*	R^2
All samples (n = 12)	r = 0.977 (p = 0.01)	y = 1.35x + 0.23	p<0.001	0.95
Analysis –excluding Crop 104 (n = 11)	r = 0.700 (p = 0.05)	y = 1.34x + 0.24	p=0.017	0.43

Analysis of the 1999 data set demonstrated that there was a significant correlation between predicted and actual *Botrytis* rot in storage (Table 4.3), even when the outlying data point (Crop 104) was removed. Linear regression analysis demonstrated a high R^2 value (accounting for 95% of the variation in the results) when all data points were used. When the outlying data point was removed, the regression analysis resulted in a R^2 value which only accounted for 43% of the variation in the data points. However, in terms of the y intercept and slope (Table 4.3), the linear regression equation derived when the outlier was excluded form the analysis was close to that of the full data set.

4.3.2 2000

During the 2000 season, coring was performed on 14 crops. To determine the effect of coring at different times after lifting, five crops were cored two or three times after lifting (Table 4.4).

Table 4.4: *Botrytis allii* detected in core samples and in bulbs after storage (2000 season).

Crop	*Date Lifted*	*Cores*			*Bulbs*	
		Weeks from lifting to coring	# Cores sampled	% cores with *B. allii*	# Bulbs stored	% *Botrytis* bulb rot
404A	6/1/00	2	360	0.0	413	4.1
404B	6/1/00	2	389	2.6	407	15.2
309	11/1/00	4	509	25.1	482	4.8
		8	491	10.6	545	6.2
Moores	6/1/00	5	590	23.4	551	15.8
		9	557	47.8	581	16.9
		13	553	46.8	555	17.3
307 {L}[1]	11/1/00	5	535	3.2	568	9.1
		9	543	6.6	554	7.8
Field Trial {I}[1]	15/2/00	1	556	3.1	525	49.3
		5	548	6.6	589	30.4
317 {C}[1]	24/1/00	7	490	3.9	547	2.9
318 {F}[1]	21/1/00	7	574	2.3	636	2.5
321	30/1/00	5	554	2.2	585	8.2
410	29/1/00	7	553	0.9	609	7.7
		11	574	2.8	611	6.7
421 {A}[1]	8/2/00	6	496	0.0	524	1.1
322	29/1/00	7	580	4.5	590	9.8
331	5/2/00	7	557	0.0	624	0.0
HM Crop 1	7/2/00	5	528	1.7	537	2.6

[1] Designation of crop previously surveyed during growing season (Chapter 3), Crop I was located 1 km from crop L in a westerly direction.

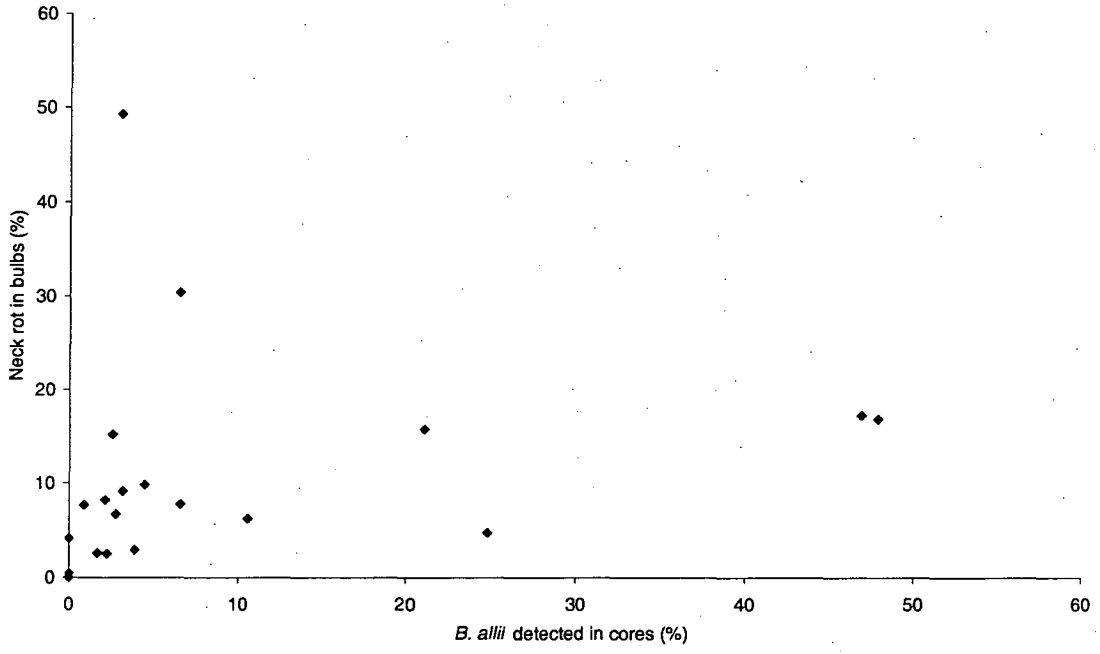

Figure 4.2: *Botrytis allii* detected in core samples and bulb rot in storage for the 2000 season.

In 2000, the incidence of *B. allii* in cores ranged from 0.0 – 47.8% and in bulbs from 0.0 – 49.3% (Table 4.4). A wider range in values occurred in the 2000 season (Figure 4.2), than in the 1999 season (Figure 4.1). Two neck core samples taken from Crop 404 resulted in an incidence of *B. allii* of 0.0 and 2.6%, indicating that the coring technique was not always able to detect bulb infection.

Table 4.5: Correlation coefficients and regression analysis of the relationship between *B. allii* detected in cores and neck rot in bulbs (2000).

	Correlation coefficient and statistical significance	*Fitted linear equation*	*F-probability*	R^2
All samples (n=20)	r = 0.197, ns	y = 0.16x + 0.09	0.405	†
Removal of outliers (n=16)[#]	r = 0.767, (p=0.05)	y = 0.26x + 0.04	p<0.001	0.56

† Residual variance exceeds variance of response variate.

[#] Samples –excluding Field Trial (I), 404A and 404B (n = 16).

Analysis of the full 2000 data set demonstrated no significant relationship or correlation between predicted and actual *Botrytis* rot in bulbs (Table 4.5, Figure 4.2). However, when the data sets for the Field Trial (I) and Crops 404A and 404B were removed, the correlation was significant and the linear regression described 56% of the data. The outlying data points were considered legitimately removed, as the bulbs in those samples were not randomised before being distributed for the experiment treatments.

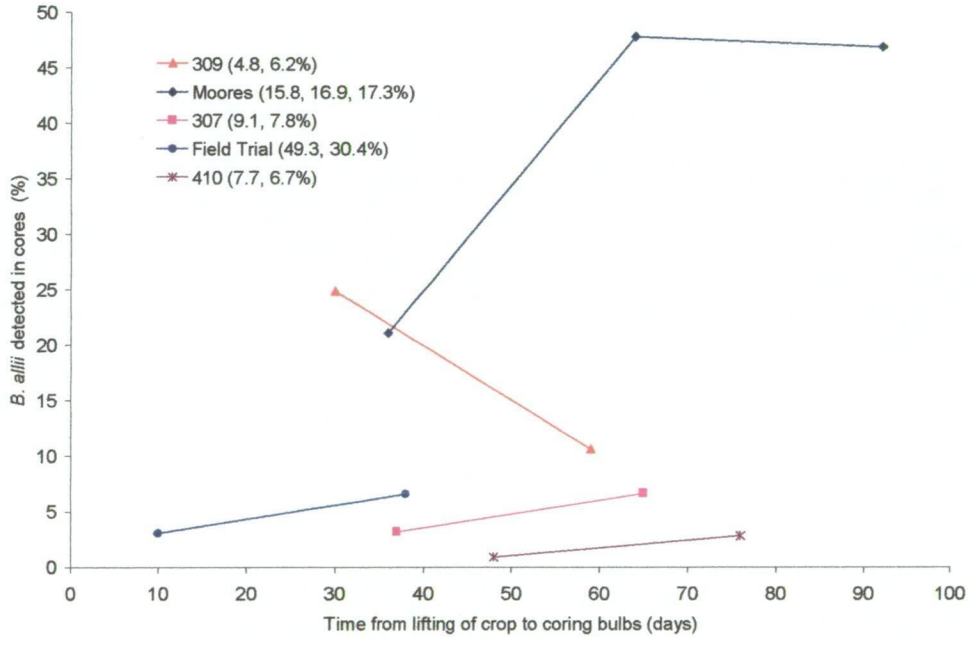

Figure 4.3: Effect of time of coring after bulb lifting on detection of *B. allii*, neck rot in stored bulbs. Numbers in parenthesis are the incidences of neck rot found in each 482-611-bulb sample after storage.

The results of coring at different intervals after lifting predominantly suggested that a higher incidence of *Botrytis* neck rot can be detected after a longer storage period between lifting of the bulbs and taking core samples (Figure 4.3). However, as neck

core samples were not replicated, it is not possible to comment on the variation around each point. For Crops 410, 307 and the Field Trial, the increase in detection of *B. allii* on bulb cores with increased duration after lifting before taking core samples, averaged 2.9% (Figure 4.3). However, in one case, Crop 309, the amount of *B. allii* detected in neck cores declined with increased time between lifting and coring, from 25.0 to 11.0%. There was an increase in the incidence detected in 'Moores' crop from 21.0% up to 47.0%. However, the actual incidence of neck rot that developed in the stored crop samples averaged 16.6%, considerably less than the predicted incidence of neck rot. Results were inconclusive and further work is needed to determine if the time between lifting and coring is important in the relationship between incidence of *B. allii* in neck cores and in bulbs after storage.

4.3.3 Analysis of both seasons (1999 and 2000)

The data for the 1999 and 2000 seasons were combined to examine the relationship between the percentage of *B. allii* detected in cores and the percentage of neck rot that developed in bulbs after a storage period (Figure 4.4).

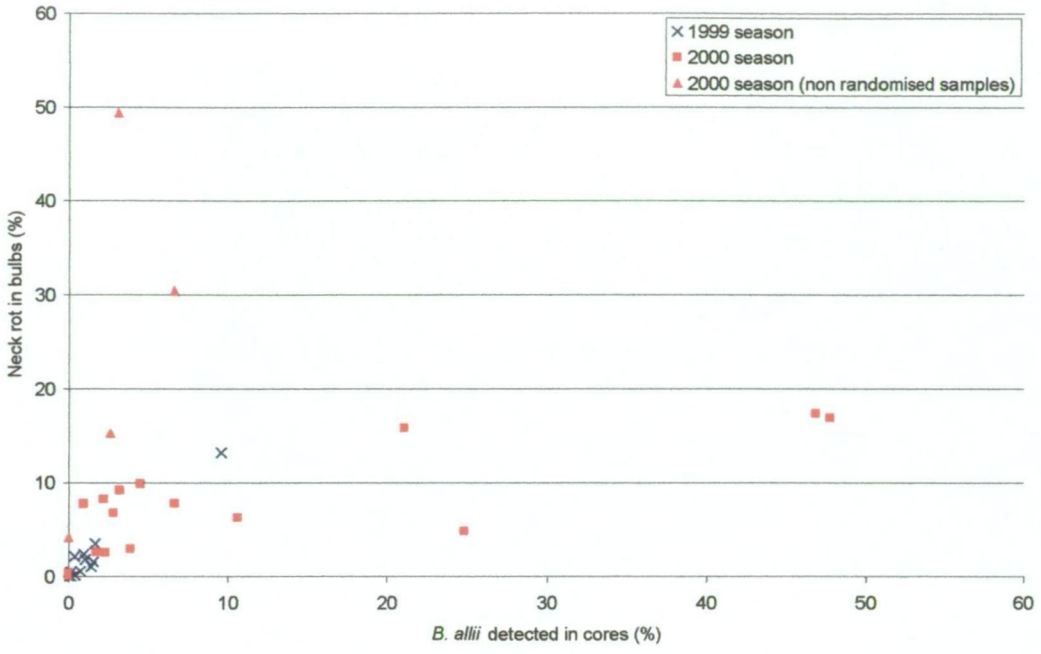

Figure 4.4: Relationship between *B. allii* detected in cores and bulb rot in storage (combined data for 1999 and 2000).

Table 4.6: Correlation coefficients and regression analysis between *B. allii* detected in bulb cores prior to storage and neck rot in bulbs after storage for the 1999 and 2000 seasons combined.

	All samples (n = 32)	*Samples excluding outliers* [1] *(n = 28)*
Correlation coefficient	$r = 0.326$, (ns)	$r = 0.773$, $p = 0.01$
Linear regression F probability and R^2	$y = 0.27x + 5.85$ $p = 0.069$, $R^2 = 0.07$	$y = 0.32x + 2.94$ $p = 0.001$, $R^2 = 0.58$
Exponential regression F probability and R^2	$y = 14.49 + (-15.33*0.63^x)$ $p = 0.007$, $R^2 = 0.24$	$y = 13.85 + (-13.20*0.87^x)$ $p < 0.001$, $R^2 = 0.68$

[1] excluding Field Trial (i and ii), 404A and 404B

The correlation between incidence of *B. allii* in cores and neck rot in bulbs was not statistically significant when all of the data points for 1999 and 2000 were used (Table 4.6). However, following collection of the bulb samples the bulbs were not randomised in the Field Trial (I) nor in Crops 404A and 404B and this may have skewed the data if highly infected bulbs were aggregated within the samples. When

data from these crops was removed from the data set, the correlation was highly significant and the relationship between *B. allii* detected on cores and neck rot in stored bulbs was improved (Table 4.6). The linear regression described 58% of the variation. The exponential regression described the data set better than the linear regression (after removal of the erroneous data), with R^2 values of 0.68 and 0.58, respectively.

4.3.4 2001 season – comparison of neck and entire core samples as predictors of neck rot in storage

There was a significant, linear relationship between the percentage of neck cores or entire cores on which *B. allii* was observed and the percentage *Botrytis* rot that developed on bulbs in storage (Figure 4.5). Each point in the graph consisted of a sample of 40 randomly-selected onions sampled to predict the level of *B. allii* on the bulbs in storage, by remaining either a neck core or an entire core, and a sample of 40 onions which were cut 120 days after lifting to determine the actual level of neck rot that developed in storage.

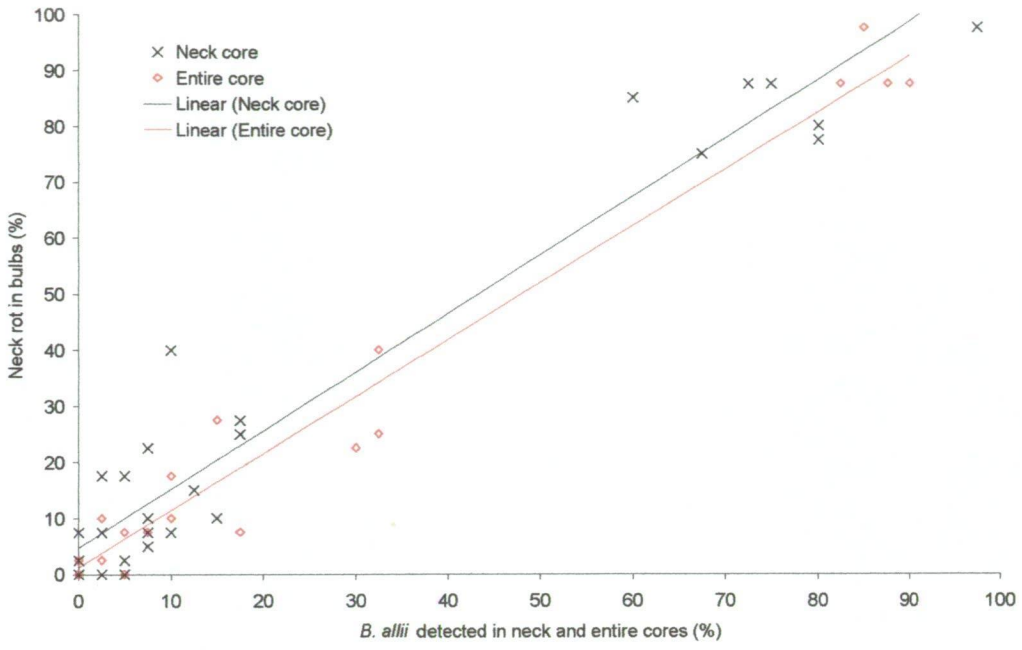

Figure 4.5: Relationship between *B. allii* detected in neck cores or entire cores of onion bulbs and incidence of *Botrytis* in neck rot in bulbs after storage. Each point is a mean of 40 randomly-selected bulbs cored for detection of *B. allii* and a paired sample of 40 bulbs stored and assessed for neck rot development.

Table 4.7: Linear regression models fitted to the coring procedure data set

Analysis method	*Regression models fitted to:* *Actual rot = a + b(x)*	*F prob.*	R^2
Data is described by 1 model	Actual rot = 0.0325 + 1.0328 * All (neck and entire cores) predicting	< 0.001	0.942
Described by two parallel lines (same slope)	Actual rot = 0.0504 + 1.10319 * Neck core predicting Actual rot = 1.0319 * Entire core predicting	< 0.001	0.945
Described by two lines, with individual slopes	Actual rot = 0.0471 + 1.0444 * Neck core predicting Actual rot = 0.0125 + 1.0147 * Entire core predicting	< 0.001	0.944

A single regression line described the data set as effectively as separate lines for each of the coring treatments (Table 4.7). This suggested that there was no significant difference between the predictive ability of the two coring methods. In addition, t-tests were used to demonstrate that there was no significant difference between the predictive ability of cores taken from the neck region of bulbs and cores through the entire onion bulbs (Table 4.8).

Table 4.8: t-tests of linear regression estimates

Actual rot = a + b(x)	*estimate*	*s.e.*	*t (df = 44)*	*t-probability*
a[1] (neck cores)	0.0471	0.0199	2.36	0.023
b[2] (neck cores)	1.0444	0.0481	21.72	< 0.001
Difference between: a (entire cores) – a (neck cores)	-0.0346	0.0307	-1.13	0.265
Difference between: b (entire cores) – b (neck cores)	-0.0297	0.0744	-0.4	0.691

[1,2] linear equation components, 'a' = constant and 'b' = slope of the line.

The first two t-tests [a (neck) and b (neck)] demonstrate significant differences from zero (Table 4.8), proving that the regression line for the neck-cored samples did not pass through the origin, and that the slope was significant. The last two t-probabilities were performed on the difference between the regression analysis describing the data sets with two different lines. This analysis demonstrated that there were no significant differences between the regression models for neck cores and entire cores, i.e., the differences between the models were not significantly different from zero.

4.3.5 2001 season timing of coring (after lifting)

There was no significant difference in the incidence of *B. allii* detected between the times of coring or the actual bulb rot in storage for bulbs obtained from Bed 1 which had a high incidence of foliar infection by *B. allii* (Table 4.9). However, for bulbs sampled from Bed 2, there were significant differences among the treatments. The percentage of cores on which *B. allii* was detected for the first time of coring was significantly lower than that of the second and third times of coring. There was no statistically-significant difference between the percentage of cores with *B. allii* at any time of coring and the percentage bulbs with *Botrytis* rot after storage (Table 4.9).

Table 4.9: Incidence of *B. allii* in neck cores sampled three different times after lifting and in bulbs after storage, for samples taken from two beds of onions, one with a high and one with a low incidence of *B. allii* detected on leaf samples..

	Bed 1	*Bed 2*
Storage sample (120 days)[1]	79.4	11.3 ab[2]
1st coring (35 days)[1]	71.9	8.1 a[2]
2nd coring (63 days)[1]	77.5	17.5 b[2]
3rd coring (91 days)[1]	78.1	17.5 b
F-probability	0.662	0.048
lsd (p = 0.05)	na[3]	7.68

[1]time after lifting.

[2]significant groups, based on least significant difference demonstrated with letters, 'a' significantly different from 'b', 'ab' = no significant difference.

[3] na = not applicable.

4.4 Discussion

4.4.1 Discussion of 1999 and 2000 seasons

During the 1999 season, the incidence of *B. allii* detected in neck cores taken shortly after lifting was a good predictor of the incidence of rot that developed in storage, but the relationship was heavily biased by 1 crop with a high incidence of *B. allii*. During 2000, several crops had a high incidence of infection, which allowed a greater spread of data points. However, the results obtained during 2000 were also more variable than in 1999, with a poor correlation coefficient and R^2 value indicating a poor relationship between predicted and actual neck rot. A combination of the 1999 and 2000 season data was best described by a linear regression. However, this described only 58% of the variation in the results, which is not considered sufficient for predictive purposes. Under ideal conditions, a 1:1 linear relationship would describe the data. However, this did not occur, and may be due to many possible factors:

Under-estimation of bulb rot:

- The neck coring procedure may have failed to sample sufficient tissue from the bulb, and hence missed latent infections which may have resided around the shoulder and base of the bulb during incubation.
- Conditions during sample preparation may have killed or inhibited *Botrytis* development in the sample tissues. For example, neck cores in trays were sometime overrun with contaminating microorganisms, making it difficult to detect *B. allii*.

Over-estimation of bulb rot:

- Cross contamination of samples might occur during coring. Spores of *B. allii* present in the air, on workbenches or bulb skins may be transferred to cores resulting in false positives.
- Coring may initiate some latent infections that do not express under bulb storage conditions, to become symptomatic, although there is no evidence to support this hypothesis.

Extending the time between lifting and coring would be expected to increase the level of detection of *B. allii* in core samples. However, although this occurred in some samples, the effect was not consistent. Results were inconclusive and further work would be needed to determine if the time between lifting and coring is important in the relationship between incidence of *B. allii* in neck cores and that in bulbs after storage.

For Crops 309 and Moores, the incidence of *B. allii* detected in the cores was much higher than the actual incidence of neck rot in stored bulbs. In Crop 309, a higher incidence of *B. allii* was detected at the first time of coring and a lower level found in a later coring sample. It is possible that when there is a higher incidence of infection in the bulb samples, there is a greater chance of spread of *B. allii* onto the skins of adjacent healthy bulbs or into the air in the room, subsequently infecting neck cores and resulting in the over-estimation of the incidence of infected bulbs. Variability in the amount of *B. allii* detected in neck cores may be influenced by a few bulbs heavily infected by *B. allii* and bearing a lot of conidia. This might result in the bench surfaces and bulbs becoming contaminated with *B. allii* conidia. To avoid this, an

effort was made to core obviously-infected bulbs at the end of the coring period. However, this precaution may not always have been sufficient.

Replicated bulb samples demonstrated little variation (around 2%) in the detected incidence of neck rot that developed after the storage period. An exception to this was the final assessment of bulbs from the Field Trial crop, where the difference in incidence of bulb rot in storage between samples was nearly 20%. This may be due to lack of randomisation of onion bulbs sampled from the Field Trial crop. Randomisation was practised for bulbs collected from all other crops for the 2000 season, except for 404A and 404B. If there were any 'hot spots' of infection in a crop and the infected bulbs were grouped, a lack of randomisation may have lead to one of the treatments having a greater or lesser proportion of infected bulbs, resulting in over- or under-prediction of neck rot in storage.

4.4.2 Discussion of 2001

Results for the coring experiments in 2001 demonstrated that the coring procedure is capable of accurately predicting the percentage of bulbs that will develop neck rot under controlled conditions. The removal of variation among crops sampled probably assisted greatly in improving the relationship between predicted and actual rot. Factors such as cultivar, time of planting and cultural practices may influence how disease develops in the bulb, resulting in differences between detectable and actual neck rot. The pathotype, source of infection, time of plant infections and weather conditions may also have a strong influence upon results. The use of a laminar flow cabinet may have improved the relationship further through removal of potential sources of cross-contamination. However, this procedure was time-consuming and unlikely to be practical for a commercial test.

There was no significant difference in incidence of *B. allii* among times of coring and after storage of bulbs sampled from a bed (Bed 1) with a high incidence of *B. allii* leaf infection. However, in a second bed (Bed 2) with a lower incidence of leaf infection, a higher incidence of *B. allii* was detected in neck cores with increased time of coring after lifting. The difference in significance of the times of coring between Beds 1 and 2 may be due to the method of infection of *B. allii* in the two beds. Bed 1 was sprayed (applied 138 days after sowing, 50 days prior to lifting, Table 8.2) with a spore suspension of *B. allii* and Bed 2 was infected solely by secondary spread of the fungus from bed 1. The data suggests that a longer period between lifting and coring will increase the level of *B. allii* found in neck cores.

4.5 General discussion

The presence of saprophytes may reduce the detection of *B. allii* by cultural methods, as a percentage of the tissue samples incubated may be overrun by saprophytes before the slower-growing *B. allii* is detected. On tests in agar cultures, Walker and Maude (1975) found that *Clonostachys rosea* produced substances which inhibited the growth of *B. allii* hyphae, and *B. allii* spores failed to germinate on agar when mixed with those of *C. rosea*. Techniques such as enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), which detect infections directly, and do not depend on visual detection of *B. allii* may, therefore, have advantages over cultural techniques of detecting latent *B. allii* infections.

Using ELISA, Linfield *et al.* (1995) indicated the progression of the fungus around the neck of the bulb might not be uniform in the early stages of infection. Levels of infection were also determined in bulbs earlier after harvest (lifting) by ELISA than by conventional isolation onto agar media. However, at the end of a storage period the

detection results using conventional isolation techniques were similar to those of the ELISA. Cultural techniques rely on mycelia or spores of *B. allii* present in the tissue sample to be cultured. The sensitivity of the ELISA suggested that *B. allii* infection could be detected in front of the advancing mycelia or spores in a bulb (Linfield *et al.*, 1995).

In designing a sampling procedure for commercial use, the size of the sample and the method of obtaining a representative sample from onion crops would need to be investigated further.

Onion neck rot not only causes direct losses to growers/packers by infecting bulbs, but also affects the onion industry in other ways. The need to hold suspect crops for a time before re-grading increases the costs of production. Crops that appear to be free from neck rot, but develop the disease in transit, can adversely affect the reputation of the onion industry's product and, thereby, affect future sales. Developing a method to determine accurately the incidence of *B. allii* latent infections in onion bulbs, would be an invaluable tool for managing this problem.

Chapter 5

5 Survival of infective propagules of *B. allii* in Tasmanian soils

5.1 Introduction

The ability of a pathogen to survive in the absence of the crops can have a major bearing on the incidence and severity of disease in succeeding crops. Experiments were conducted to determine the ability of *B. allii* to survive in the absence of its host, as sclerotia, conidia in soil and on plant debris.

Sclerotia are formed by fungi for long-term survival during periods not conducive to growth. They are formed in response to a lack of host material or unsuitable environmental conditions. Sclerotia of some fungi (e.g., *Verticillium dahliae*) may remain viable in soil for 10 or more years (Brown, 1997). The only publication on sclerote survival of *B. allii* comes from Britain, where Maude *et al.* (1982) reported that in non-sterile soil, *B. allii* sclerotia were non-viable within 6 months. This suggests that the current practice in the Tasmanian onion industry of planting fields with onions 1 year in every 4 years is sufficient to prevent the pathogen being carried over between onion crops. However, no information is available on the survival of *B. allii* sclerotia in Australia. Sclerotia survival times may be different between the UK and northern Tasmania due to climate, soil microbiology, cropping practices etc, likewise differences may occur in *B. allii* sclerotial survival throughout Australia.

Although reports have been published on the use of selective media for assaying *B. allii* from soil (Lorbeer and Tichelaar, 1970; Kritzman and Netzer, 1978), no studies on survival of *B. allii* conidia in soil have been conducted.

In one report, Tichelaar (1967) demonstrated that *B. allii* could survive saprophytically on decaying plant materials of crops other than *Allium* spp. (cereals, lucerne, bean and pea). One of the objectives of the work described in this chapter was to evaluate the ability of *B. allii* to survive saprophytically on poppy (*Papaver somniferum*), a common crop grown in rotation with onion in northern Tasmania.

5.2 Materials and Methods

5.2.1 Experiment 1 – sclerotial survival experiment

Sclerotia were collected from onions exhibiting neck rot after a storage period of 3 months in a "fantainer" (container with forced ambient air flow). The onions were collected from a packing shed in June 1999 and sclerotia were removed from the onions during July 1999. The sclerotia were separated into groups so that each group contained a similar size range of sclerotia. Sclerotia were assessed at the beginning of the experiment to determine the percentage viability (as described later). Twenty sclerotia were placed into each of 15 nylon mesh bags that were 10 x 10cm in size. The bags were constructed and sealed using a soldering iron to fuse the material. The sclerotia in nylon mesh bags were laid into 20 cm diameter pots half-filled with soil, 1 mesh bag per pot, and 6 cm of sieved (5 mm - mesh size of sieve) soil was placed on top of the mesh bags. The pots had large drainage holes in the base. Soil was red ferrosol (kraznosem) collected from the same location as Field Trial 1 (Chapter 7), from the University of Tasmania farm, in Burnie. The field from which soil was

collected had not had onion crops for over 4 years. The pots were then buried down to soil level in a pasture soil at Burnie, Tasmania, to mimic (as closely as possible) a natural soil environment.

Three replicate bags containing 20 sclerotia each were retrieved at each of four times after burial. The experiment was commenced on the 16/8/99 and sclerotia were retrieved at intervals for up to 879 days after burial (Table 5.1). The experiment was arranged as a completely randomised design and was analysed by ANOVA with Genstat® statistical package, with means compared using least significant difference.

Table 5.1: Schedule of sclerotial survival experiment 1

Harvest #	Assessment date	Days
0	16/8/1999	0
1	14/9/1999	29
2	18/10/1999	63
3	10/1/2000	147
4	11/1/2002	879

After retrieval, sclerotia were removed from the nylon bags, washed in 100 ml of deionised water, dried on paper towels and placed onto 1.0% water agar in petri plates. Plates were incubated in the dark at 20° C for 5 days, then assessed for germination using a dissecting microscope. Sclerotia were incubated and assessed after an additional 5 days to check for any sclerotia that were slow to germinate. The number of sclerotium pieces that were viable was calculated as a percent of the initial number of sclerotia that were placed into the bags. For the number of sclerotia that were recovered, the number of sclerotium (particles excluding pieces smaller in diameter than 0.5 mm) were counted.

5.2.2 Experiment 2 – sclerotial survival experiment

In this experiment the survival of sclerotia from naturally infected onions and from sclerotia cultured in the laboratory was assessed. To produce sufficient sclerotia for experimental purposes, sclerotia were produced artificially on a bran substrate similar to the method of Clarkson *et al.* (2000) for the production of sclerotia of *Botrytis squamosa*. Ten grams of unprocessed natural bran and 250 g of sand (< 500 µm diameter particles) were placed into a conical flask and autoclaved at 121 °C for 20 minutes (100-130 kpa). *Botrytis allii* (isolate C3, Appendix 12.9) was grown on pectin agar until sporulation. A piece of agar (1 cm^2) with conidiophores was shaken in 60 ml of sterile water and then poured into the conical flask containing the bran/sand mixture in a laminar flow cabinet. The flask was capped with aluminium foil and incubated in the dark at 20°C for 6 weeks. The contents of the flask were then wet-sieved through a 500 µm (mesh size) sieve to separate the sclerotia from the sand. Sclerotia were then dried in a laminar flow cabinet and separated from the bran particles. Sclerotia ranged in size from 1 – 3 mm in diameter.

Sclerotia were collected during May 2000 from diseased onions used in the predictive experiments (described in Chapter 4) carried out during 1999/2000. Sclerotia ranged in size from 1-10 mm in diameter.

For the bran and onion-derived sclerotia, 25 and 30 sclerotia respectively, were placed in nylon mesh bags. The nylon mesh bags were then buried in pots of soil as described for Experiment 1 (i.e., 6 cm depth in pots, which were buried in the field in a pasture soil on the University of Tasmania farm, Burnie). One treatment each of laboratory-produced sclerotia and naturally-produced sclerotia were stored in a laboratory

cupboard (15 – 20°C) in the dark, and assessed for viability after 554 days. The experiment commenced on the 6/7/00 and four replicate bags per treatment were retrieved at intervals (Table 5.2). Sclerotia maintained in the laboratory were placed between fleshy scales of an onion bulb in an attempt to break possible dormancy of the sclerotia.

Table 5.2: Schedule of events for sclerotial survival experiment 2

Dates	Days from commencement	Harvest interval number	
		Sclerotia from onion	Sclerotia from bran
6/7/00	0	1	1
11/10/00	97	2	-
9/1/01	187	3	2
26/4/01	294	4	-
9/7/01	368	5	3
10/10/01	461	6	-
11/1/02	554	7	4

To assess viability, sclerotia were removed from the nylon mesh bags and were placed directly onto 1% water agar in Petri plates. The same methods of incubation and assessment were used as described in Experiment 1. The sclerotia from naturally infected onions and the sclerotia grown on bran were arranged in completely randomised designs and were analysed with ANOVA using the Genstat® statistical package, with differences between means of percent germination and recovery determined using least significant difference.

5.2.3 Conidial survival in soil

Kritzman's agar efficacy

A test of agar media was conducted to ensure that Kritzman's agar was not inhibitory to the germination of *B. allii* spores. A spore suspension was made from onions that

had been injected 7 weeks prior with isolate C3 of *B. allii,* as described in Appendix 12.3. A comparison was made between 0.5% water agar, Pectin agar and Kritzman's agar with 3 replicates per treatment (Appendix 12.5). A 0.5 ml spore suspension in sterile water was spread over each plate with a bent glass rod. The plates were dried in a laminar flow cabinet, sealed with clingwrap and placed into an incubator at 21°C in the dark. After 16 hours incubation, between 249 and 302 spores were counted per plate. Counts of germinated and ungerminated spores were made at 200X magnification. A germinated spore was defined as a spore with a germ tube of equal or greater length than the spore. The experiment was organised as a completely randomised block design. The percentage spore germination was calculated and the results tested with analysis of variance using Genstat® and differences between means were tested with least significant differences.

Conidial survival in soil

Survival of *B. allii* conidia in soil was tested in a red Ferrosol soil obtained from a cropping paddock, which had not had onions for at least six years. Soil was air-dried in the laboratory and sieved through a 1 mm (mesh size) sieve. A spore suspension of *B. allii* was made from conidiophores growing on an onion inoculated (Appendix 12.2) with isolate C3 (Appendix 12.9). The number of spores per ml of spore suspension was estimated using a haemocytometer. The experiment consisted of three different concentrations of spores and one control (soil only) with two replicates of each. Each conidial suspension was inoculated into 50 g of soil, made up to 80% moisture content and mixed into a slurry in a 100 ml vial. At 0, 6 and 12 days, a 1.0 gram sub-sample of wet soil was made into a dilution series of 10^{-1} to 10^{-5}. The dilution series was made by placing the sub-sample into a 15 ml vial with 9 ml of

sterile dH_2O, shaken and then a 1.0 ml aliquot of that dilution was mixed into another 9 ml of dH_2O. This process was repeated to a dilution of 10^{-5}. A 1.0 ml aliquot from each of the dilutions was spread onto Kritzman's agar plates with a bent glass rod, under aseptic conditions and the plates left to dry free of surface water in the laminar flow cabinet. The petri plates were then incubated in the dark at 20°C for 10 days before being assessed for the total number of colonies of *B. allii*. The number of colonies of *B. allii* was identified using a dissecting microscope (40X). The experiment was arranged as a randomised complete block design, with 3 concentrations of conidia and 3 times of assessment, with 2 replicates per treatment combination. The experiment was analysed by Genstat® statistical package as a two-way ANOVA.

5.2.4 Alternative hosts and competitive saprophytic ability of *B. allii*

The potential for *B. allii* to live saprophytically on debris of poppy (*P. somniferum*) crops was assessed. Poppy crops are grown widely in rotation with onions in Tasmania and considerable poppy residue is left in paddocks. An experiment was performed to determine if *B. allii* conidiophores could be produced on dead poppy trash. Dry poppy trash was collected from a field, and 10 g quantities placed into 500 ml conical flasks. Half of the flasks containing poppy trash were autoclaved at 121°C for 20 minutes (100-130 kpa) to sterilise the trash. The others were left non-sterile. A spore suspension of *B. allii* was made by shaking a 1 cm^2 piece of agar from a sporulating pectin agar culture of *B. allii* (isolate C3) in 10 ml of sterile dH_2O. An aliquot (10 ml) was then pipetted into each flask containing the poppy trash. The flasks were incubated at 20°C in the dark and were examined every day for the first week of incubation and periodically for a period of 6 months, for the presence of *B.*

allii conidiophores and sclerotia. There were four replicates of both treatments (sterile and non-sterile poppy trash) arranged in a completely randomised design. No statistical analysis was undertaken.

5.3 Results

5.3.1 Experiment 1

All sclerotia produced conidiophores bearing conidia shortly after germination (Figure 5.1). Mycelial germination (where no conidiophores are produced) was not noted. In August, September and October 1999, 100% of sclerotia were recovered, with the viability not significantly different among retrieval dates, with 72, 73 and 75% of sclerotia viable, respectively. In January 2000, the percentage viable sclerotia was 63% and the recovery of sclerotia was 88%, both of which were not significantly different from previous periods of retrieval. By January 2002, after 29 months of burial, the percentage recovery was 73%, which was not significantly different from previous times of retrieval of sclerotia. The percentage of sclerotia viable was 13%, significantly lower ($P = 0.05$) than previous sampling times (Figure 5.2).

Figure 5.1: Germinated *B. allii* sclerotium with conidiophores and conidia (20X magnification).

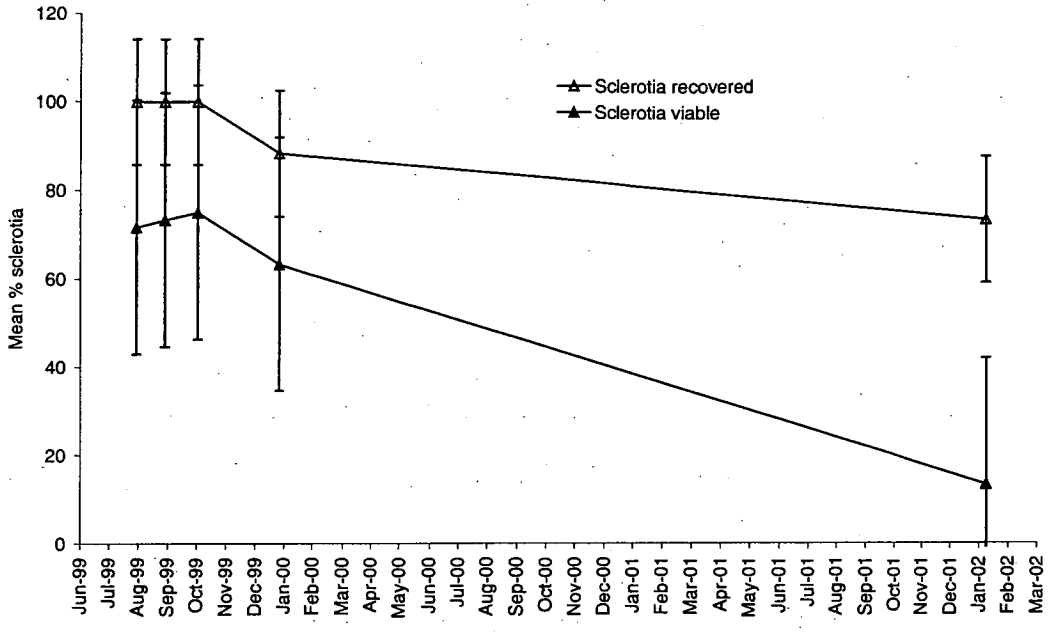

Figure 5.2: Recovery and viability of *B. allii* sclerotia from onion after burial in soil for up to 29 months. Bars represent least significant difference values (P=0.05) between means at each time period.

5.3.2 Experiment 2 – sclerotial survival experiment

The percentage of laboratory-produced sclerotia recovered between July 2000 and January 2002 ranged between 73%-100% and did not differ statistically. However, the percentage of viable laboratory-produced sclerotia declined significantly (P = 0.05) from 100% in July 2000 to 24% in January 2002 (Figure 5.3).

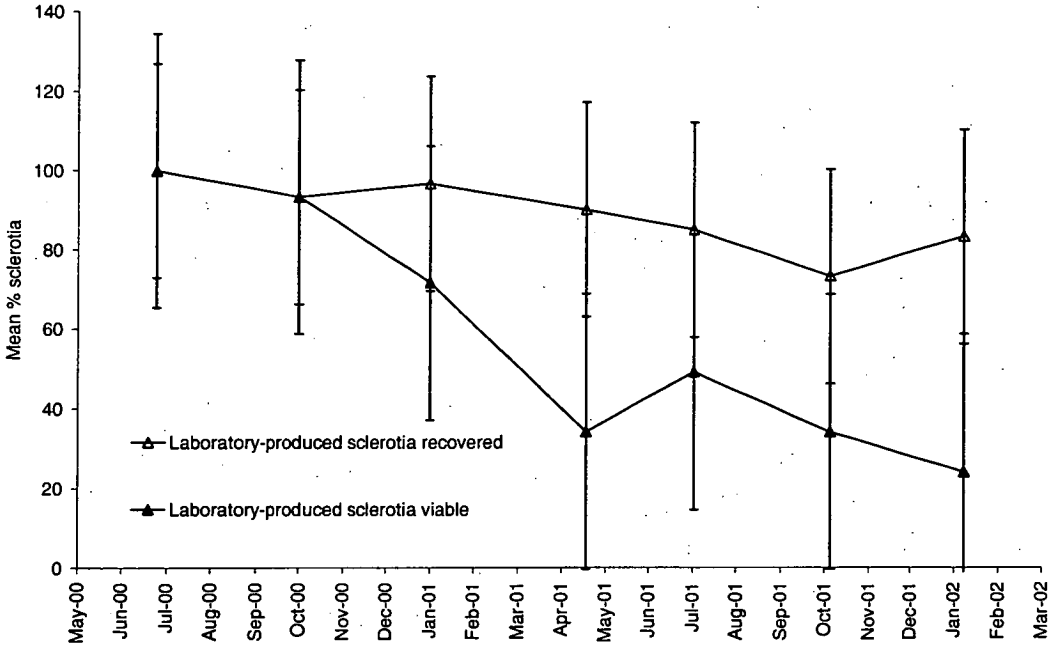

Figure 5.3: Recovery and viability of sclerotia produced from bran cultures of *B. allii* after burial of sclerotia in soil for different periods. Bars represent least significant difference (P=0.05) between means.

The recovery of sclerotia originally collected from infected onions (Figure 5.4) rose to 156% of the original number buried at July 2001 and subsequently declined to 75%. However, these differences were not statistically significant. The numbers of viable sclerotia recovered ranged from 70% (June 2000) to 0% (July 2001) but the differences among recovery periods were not statistically significant (Figure 5.4).

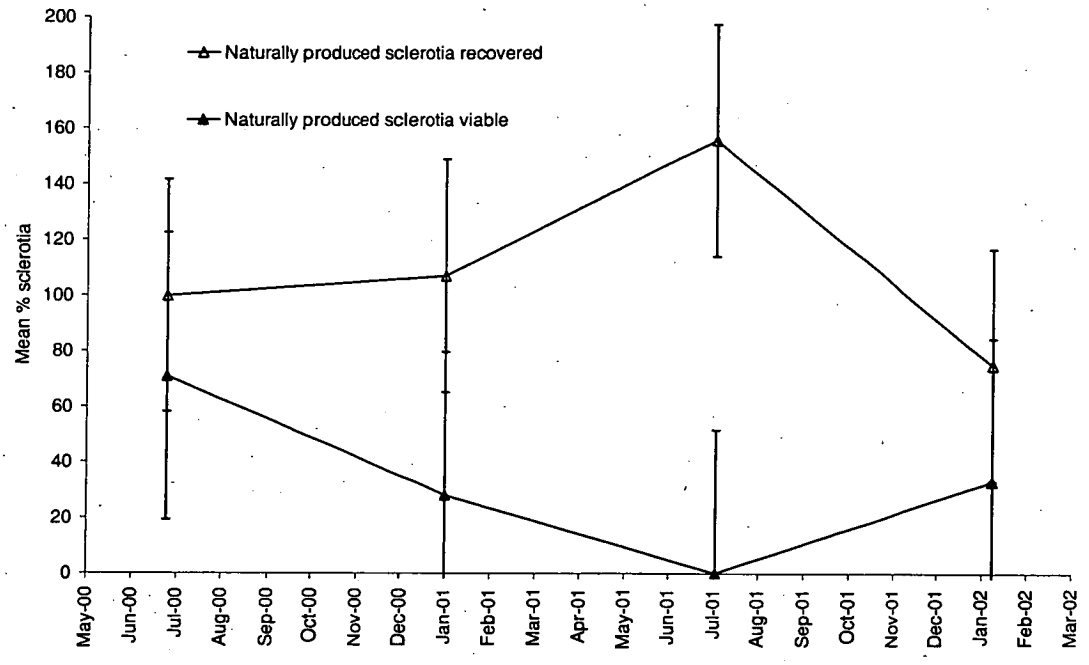

Figure 5.4: Recovery and viability of naturally-produced sclerotia of *B. allii* after burial of sclerotia in soil for different periods. Bars represent least significant difference between means (P = 0.05).

Laboratory-stored sclerotia

After 554 days storage in the laboratory in the dark, none of the 100 sclerotia derived from infected onions, and only 1 of the 120 sclerotia produced on bran, germinated. Sclerotia derived from infected onions were all heavily contaminated with saprophytes (Table 5.3). Sclerotia produced on bran did not have any contaminants after the laboratory storage period. Sclerotia from bran cultures did not germinate when placed between onion scales in an attempt to break potential sclerotium dormancy.

Table 5.3: Fungal contaminants recovered from laboratory-stored naturally-produced sclerotia (out of 25 sclerotia per replicate).

Fungal spp.	*Replicate*			
	1	2	3	4
Penicillium	22	20	19	20
Unidentified ascomycete	2	3	6	3
Rhizopus spp.	2	2	0	2
Other	0	2 sterile fungi	0	2 unidentified fungi

Numbers in table indicate the number of fungal contaminants per the 25 sclerotia of each replicate. The total number of contaminants may equal or be more than the number of sclerotia per replicate, as some sclerotia yielded more than one contaminant.

5.3.3 Conidial survival in soil

Kritzman's agar efficacy

The tests of the various agar media for potential inhibition of spore germination demonstrated there was no statistically significant difference in spore germination among different media ($P = 0.70$, LSD = 13.5%). The mean percentage of spores (250-300/plate) germinated on water agar, pectin agar or Kritzman's agar in petri plates after 16 hours incubation was 89.5%, 94.0% and 91.5%, respectively.

Conidial survival in soil

From original spore concentrations of *B. allii* of $11x10^4$, $7x10^4$ and $3x10^4$ viable spores/g of soil, viability of spores declined at an approximate linear rate with time of storage in soil. At 12 days, no viable propagules of *B. allii* were recovered (Figure 5.5). Significant difference was found between concentrations ($P < 0.001$) and times of storage ($P < 0.001$) with LSD's of 1.4×10^4 respectively, and a significant

interaction between concentration and time of storage in soil was also found (P = 0.008) with an LSD between the combinations of 2.4 x 10^4.

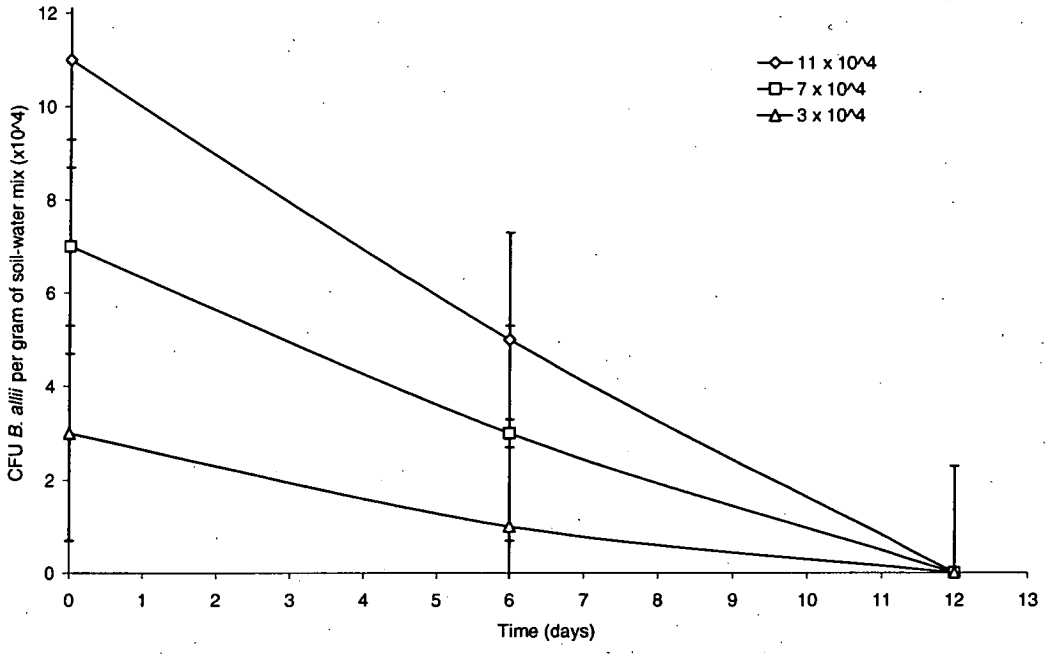

Figure 5.5: Number of *B. allii* CFUs per gram of soil at 0, 6 and 12 days after inoculation with 3 concentrations of spores. The LSD (P = 0.05) between the spore concentration and duration of storage combinations = 2.4x10^4 CFU/g soil.

The vials with the highest concentration of *B. allii* spores demonstrated significantly different mean CFUs of *B. allii* recovered at each of the three sample times. In the vials with the lowest concentration of spores, there was no significant difference between the mean number of CFU, recovered among the sample times. The vials with the intermediate concentration of conidia only had significantly different recovery rates for *B. allii* between the first and last sample times (Figure 5.5).

5.3.4 Alternative hosts and competitive saprophytic ability of *B. allii*

Conidiophores of *B. allii* were not observed on poppy trash that was inoculated but not sterilised, for all 4 replicates. However, on the sterilised poppy trash, *B. allii* conidiophores covered the trash within 4 days after inoculation (Figure 5.6) on all 4 replicates. No sclerotia were observed on the trash over the 6 months of the experiment, for all 4 replicates. ANOVA was not performed on these results.

Figure 5.6: Grey spore masses of *B. allii* on sterilised poppy trash.

5.4 Discussion

5.4.1 Sclerotial survival

In the UK, *B. allii* sclerotia were found by Maude *et al.* (1982) to be non-viable after 6 months in soil. However, under Tasmanian conditions, sclerotia of *B. allii* were found to be viable in the soil for much longer, up to 29 months. This may be due to a number of different factors such as isolate of *B. allii*, soil type, soil microbial activity and environmental conditions (soil temperature, moisture content and fluctuation in these variables).

The micro-biota of the soil, particularly the presence of hyperparasites, may affect the period of sclerotial survival. Several fungi are well-known parasites of sclerotia of other fungi, including *Coniothyrium minitans* and *Sporidesmium* spp. Walker and Maude (1975) demonstrated that *Clonostachys rosea* was a common contaminant of *B. allii* isolates taken from growing plants and onion bulbs and of *B. allii* sclerotia. *C. rosea* prevented sclerotia of *B. allii* from germinating and infected sclerotia were found to be deeply penetrated by the fungus. On tests in agar cultures, Walker and Maude (1975) found that *C. rosea* produced substances which inhibited the growth of *B. allii* hyphae, and *B. allii* spores failed to germinate on agar when mixed with those of *C. rosea.* No *C. rosea* isolates were identified on sclerotia experiments conducted in this thesis. However, a *Penicillium* spp. was isolated from many of the sclerotia stored in the laboratory. *Penicillium* spp. have been involved in the hyperparasitism of other fungal species. For example, *P. vermiculatum* has been reported to hyperparasitise sclerotia of *Sclerotinia sclerotiorum* (Liu, 1989). The *Penicillium* spp. found on the sclerotia of *B. allii* stored in the laboratory may have contributed to the low percentage of sclerotia germination. However, the sclerotia grown on bran did not yield any known hyperparasites, and they also could not be germinated. Differences in conditions leading to the formation of sclerotia and stage of decomposition of the bulbs may have a large influence upon the hardiness and survival of sclerotia. Harper *et al.* (2002) reported that sclerotia of *Sclerotium cepivorum* collected from onion crops late-season had lower viability than sclerotia collected early-season, presumably due to a longer period of exposure of sclerotia collected later in the season to fluctuating environmental conditions and microbial attack compared to sclerotia collected earlier in the season.

Sclerotia of *B. squamosa* are capable of surviving long periods in soil. Ellerbrock and Lorbeer (1977a) found that after 21 months, 83% of sclerotia buried at a depth of 15 cm were viable, as opposed to 43% of sclerotia at a depth of 3 cm. This increased survival rate at a greater depth in the soil is probably due to less extreme temperature and moisture fluctuations and/or a higher level of fungistasis compared to shallower depths in the soil (Ellerbrock and Lorbeer, 1977a). Clarkson *et al.* (2000) suggested that tillage operations in Britain during preparation of seedbeds (February and March) could bring sclerotia to the surface to germinate. Sclerotia of *B. squamosa* on the surface take 3-4 weeks to produce conidia. As bulb onions emerge approximately 4 weeks after sowing, seedlings could be exposed to conidia of *B. squamosa* immediately upon emergence (Clarkson *et al.*, 2000). If *B. allii* behaves in a similar fashion, this may be a potential mechanism for introduction of *B. allii* into crops. The sclerotial survival studies in this thesis were conducted in an undisturbed pasture soil. Sclerotia may survive for reduced periods in soil that is regularly tilled.

At the commencement of the experiment, all of the sclerotia produced on bran germinated when placed onto 1% water agar. Perhaps after remaining in a dry environment for a long period, dormancy had developed in the sclerotia and may have required breaking (i.e., with cycles of wetting and drying) to achieve germination. Brix and Zinkernagel (1992) found that sclerotia of most isolates of *S. cepivorum* demonstrated a constitutive dormancy immediately after maturing. Gerbrandy (1992) also found freshly-harvested sclerotia of *S. cepivorum* did not germinate, but dormancy could be broken in most isolates tested by a 48-hour drying period prior to incubation under moist conditions. Laboratory-grown sclerotia in this thesis study were similar in their survival characteristics to sclerotia produced on onion bulbs,

when buried in soil. For sclerotial germination of other *Botrytis* spp. favourable conditions of temperature and water potential need to occur (Coley-Smith *et al.*, 1980; Clarkson *et al.*, 2000). However, there are no reports of other required stimuli, e.g., for example dry or cold periods. The sclerotial of *S. cepivorum* are stimulated to germinate by the presence of plant exudates from *Allium* hosts (King and Coley-Smith, 1969). The *B. allii* sclerotia stored in the laboratory for 18 months that did not germinate were placed between fleshy scales of an onion in an attempt to break dormancy. However, this was unsuccessful. Wetting and drying periods could be investigated as a means of breaking the apparent dormancy of *B. allii* sclerotia. It may be useful to investigate root exudates and their effect upon breaking apparent dormancy, as these may be different to scale 'exudates'.

Sclerotia leak solutes when placed under wet conditions following a short period of air-drying (Coley-Smith *et al.*, 1980). Sclerotia can leak repeatedly if placed under successive dry and wet conditions. No reports are available for the amount of solutes leaked by *B. allii.* However, other *Botrytis* spp. appear to be intermediate in the amount of solutes they leak when compared to other fungi (Coley-Smith *et al.*, 1980). The solutes that are leaked consist of a wide range of substances, with soluble carbohydrates accounting for as much as 50% of the dry weight of material leaked and amino acids accounting for 30% (Coley-Smith *et al.*, 1980). Determining the amount and rate of solutes that *B. allii* sclerotia leak may provide answers to the longevity of sclerotia under different environmental conditions. Ellerbrook and Lorbeer (1977b) found that sclerotia of *B. squamosa* were capable of producing conidia up to four times in the laboratory by a repeating sequence of dry storage and

moist incubation. A study to determine the number of times that *B. allii* sclerotia can produce conidia may be of use in deriving disease management plans.

Maude *et al.* (1982) found the viability of *B. allii* sclerotia in onion half bulbs declined within three months of burial. Survival of sclerotia or other propagules in onion crop residues under Tasmanian conditions was not examined in this thesis and may make a useful additional study. It was demonstrated that conidia had a short survival period in soil at high moisture content. However, propagules in debris may be protected to some extent and may survive longer. Determining the length of survival of such potential sources of inoculum will assist in developing management tools.

The bags that were used to store the sclerotia for the survival studies may have had some influence upon the survival of the sclerotia. Any biocidal properties or physical barrier properties of the fabric may have influenced the results somewhat and could be tested in the laboratory. However, the use of the bags was necessary for this particular experiment and is a standard technique used in other studies such as that of Maude *et al.* (1982).

Methods are available for the enumeration of *S. cepivorum* sclerotia from soil (Metcalf, 1997). The enumeration of *B. allii* sclerotia from soil could be developed as a test for determining the significance of the sclerotia as an inoculum source. However, developing a procedure for *B. allii* may be more difficult than for *S. cepivorum* as *B. allii* sclerotia are of less uniform size and shape than those of *S. cepivorum.* As with any soil test, there may be difficulties in obtaining a sufficiently

representative sample to give an accurate assessment of sclerotia numbers and spatial distributions.

5.4.2 Conidial survival

Kritzman's agar was used by Kritzman and Netzer (1978) to isolate *B. allii* in both naturally or artificially infected soils. The use of the Kritzman's agar as a selective media in this thesis was also justified as no inhibition of conidia germination was found when compared to other media (WA and Pectin agar).

Botrytis allii conidia were found to survive for less than 12 days in saturated soil. This finding of a short survival time is similar to some findings by other researchers. Ellerbrock and Lorbeer (1977ab) reported that *B. squamosa* conidia could be recovered from soil in onion fields during leaf blight epidemics, using an amended Martin's rose bengal agar. *Botrytis squamosa* could be detected in the soil for 1-2 weeks after harvest of the crops. However, when field soil was artificially inoculated with conidia, *B. squamosa* was recovered for up to 2 months after infestation (Ellerbrock and Lorbeer, 1977ab). Repeated wetting and drying of the soil adversely affected the survival of *B. squamosa,* with no conidia recovered after two wetting and drying cycles. Conidia in soil kept in a stable state of either moist or dry survived for over 8 days (Ellerbrock and Lorbeer, 1977ab). Another method of survival of fungi is the production of microconidia. *Botrytis fabae* and *B. tulipae* produce microconidia from macroconidia when buried in soil. Microconidia appear to outlive the macroconidia, but are more difficult to germinate. Microconidia may be important for survival of fungi under adverse conditions and from one season to the next (Coley-

Smith *et al.,* 1980). No reports have been made on the production of microconidia by *B. allii.*

Botrytis conidia have been reported to survive for much longer periods out of the soil environment and on plant surfaces than in the soil, particularly where they are protected from direct sunlight. For example, Walter *et al.* (1999) found conidia of *B. cinerea* survive for up to 16 weeks in the field on the surface of Kiwifruit, where it was hypothesised they were protected from sunlight by the hairy nature of the fruit and the crop canopy. See Chapter 6 (Section 6.3.7) for evaluation of the effect of sunlight upon survival of *B. allii* conidia.

5.4.3 Alternative hosts and competitive saprophytic ability of *B. allii*

Botrytis allii conidiophores were not observed on non-sterile poppy crop residue and were, therefore, not considered to colonise the poppy residue naturally.. This is possibly due to competing microorganisms present in/on the poppy residue. However, on sterile poppy crop residue, *B. allii* was able to grow profusely. It should be noted that competitive saprophytic ability is not defined by just ability to sporulate. To determine the full nature of the competitive saprophytic ability of *B. allii* further experiments are required. Tichelaar (1967) reported that *B. allii* lived saprophytically on decaying plant materials (cereals, lucerne, bean and pea). However, this report was not substantiated with any other reports in the literature. Tichelaar (1967) did not reisolate *B. allii* from the debris it may be that other *Botrytis* spp. were misidentified as *B. allii.* It may be of value to attempt to infect poppy crop tissue that is beginning to senesce. *Botrytis allii* may be able to colonise such tissue before other saprophytes become established on the residue.

5.4.4 General discussion

Determining the minimum length of crop rotation out of onions is important for developing effective disease management protocols. Sclerotia are one means of *B. allii* overwintering and re-entering a future crop. Hence, determining the length of time these propagules survive in the soil is important in recommending suitable durations between onion crops in a crop rotation sequence. Current practice by the Tasmanian onion industry is a minimum of 4 years of rotation with other crops before a paddock is re-sown with onions. This may need to be extended to ensure that the onion crops will not be infected by conidia produced on surviving sclerotia. The potential role of poppy as an alternative host for *B. allii* was investigated. Other crops such as carrots and potatoes which are often grown in rotation with onion in Tasmania may also need to be investigated. In practice other considerations should be made such as proximity of onion crops, including both crops in the current season and proximity of fields in which onions were grown the previous year and which may contain viable sclerotia. To minimise the risk of previous crops providing such a source of inoculum, thorough clean-up of bulbs left on the ground should be done. Enhanced degradation of sclerotia might be achieved by soil amendment with parasitic fungi and/or organic materials including biofumigant crops e.g. *Brassica* spp. high in glucosinilates and cover crops (Matthiessen and Kirkegaard, 1997; Abawi and Widmer, 2000).

Chapter 6

6 Spore release and plant infection events of *Botrytis allii*

6.1 Introduction

There has been little published research on spore release and aerial dispersal of *B. allii*. Attempts by Maude and Presly (1977a) in 1972 and 1973 at trapping air-borne *Botrytis allii* spores with a Burkard volumetric spore trap were unsuccessful, even though conidiophores of *B. allii* were observed in the onion crop. They concluded that the spores of *B. allii* were windborne, as they observed disease spread within plots and from plots established with *B. allii*-infected seed to plots that were established with clean seed (Maude and Presly, 1977ab). In one trial, they hypothesised that the fungus had spread within a season from an infected plot to an isolated plot 270 m away (Maude and Presly, 1977a). In another experiment, they noted *B. allii* had spread from an infected plot to a healthy plot at a distance of 137 m (Maude and Presly, 1977b). From this, Maude and Presly (1977a) stated that "*B. allii* was thus source specific and not ubiquitous in onion crops and restricted in its range of spread to a distance of about 270 m". It was also reported by Maude and Presly (1977a) that *B. allii* progressed more rapidly during wet humid conditions than during dry conditions (1972 vs. 1973, respectively).

Little has been published on dispersal gradients for *Botrytis* spp. However, a lot of work has been done for other fungal species. Dispersal models for these species are likely to apply to the *Botrytis* genus and other particles of similar dimensions and properties (see literature review Section 1.4.6).

Much information has been published on spore release and disease modelling and forecasting for *B. squamosa* (literature review Section 1.9.2). Spores of *B. squamosa* are reasonably distinct from other *Botrytis* spp. and the pathogen causes visible, distinctive lesions on leaves of the onion plant (Schwartz and Mohan, 1995). The maximum numbers of conidia are caught during periods of decreasing relative humidity and increasing temperature (Lorbeer, 1966; Small, 1970; Swanton, 1977).

Information on the spore release and dispersal of *B. allii* will assist in understanding the epidemiology of this fungus, and may be critical in developing disease monitoring procedures and disease control strategies. A series of experiments was conducted to determine the influence of weather parameters on spore release and dispersal of *B. allii*. Novel spore trapping techniques were also tested for use in spore trapping experiments. *Botrytis allii* spores are difficult to distinguish from background material trapped on spore trapping surfaces and, possibly, from the spores of *B. cinerea*. Shirane *et al.* (1989) used a nuclei staining procedure to differentiate *B. allii* from *B. byssoidea*, proving by the difference in the number of nuclei per conidia that they were not conspecific. This stain was examined as a tool to assist in identification of *B. allii* spores.

6.2 Methods

6.2.1 Effects of humidity, free water and spore concentration on spore germination

To determine the effects of free water and spore concentration on spore germination the following experiment was performed. A spore suspension was made from an onion that had been injected 3 weeks prior with spores of isolate C3 of *B. allii* (Appendix 12.9). The concentration of spores in the solution was determined to be 1.6 x 10^6 spores per ml using a Haemocytometer. A 0.2 ml aliquot of the spore suspension was plated onto each of 6 Petri plates containing water agar (WA) and a 0.5 ml aliquot on each of another 6 Petri plates of WA. Suspensions were spread over the agar surface with a bent glass rod, to give concentrations of 3.2 x 10^5 and 8 x 10^5 spores per plate, respectively, with 6 replicate plates of each spore concentration. Plates were allowed to dry in the laminar flow cabinet for 30 minutes. Petri plates with the spores were incubated (with lids on) at 20°C in the dark, then assessed at 200 X magnification for the number of spores that had germinated after 3.5, 16.5 and 23.0 hours. The definition of a germinated spore was a spore that had a germ tube as long as, or longer than, the length of the spore. At least two hundred spores were examined per plate. The experiment was set up as a completely randomised design and analysed by ANOVA using Genstat® statistical package with mean separation by least significant difference.

An experiment was performed to determine if *B. allii* spores would germinate in the presence of high humidity, without free water. Spores from an onion were dusted onto

a slide, which was then placed into a 1 L food container with a lid containing a wet tissue to provide high humidity. The slide was held above the wet tissue on plastic mesh. The apparatus was incubated at 20°C in the dark. The slide was removed after 24 hours and examined for germinated spores. This experiment was repeated, leaving the slide in the container for 48 hours. It was noted that condensation had formed on the lower side of the slide. The experiment was repeated twice, and each time 100 spores were assessed for germination using the microscope.

6.2.2 Quest spore trap – volumetric spore trap

Volumetric spore trapping was undertaken using a Quest spore trap (Figure 6.1), distributed by Melpat International, Melbourne. The Quest trap samples 20 litres of air per minute, and is powered by a rechargeable 12 V battery, which is kept charged by a solar panel. A regulator controls the power unit. The supply voltage is 9-15 V D.C. and the supply current is 200 mA. The spore trap swivels on an axle and a windvane ensures that it faces continuously into the wind. The trap orifice is protected from rain by a shield. The orifice stands 0.5 m above the ground and is 14 mm long by 2 mm wide. The distance between the orifice and the collection surface is 1.0 mm. Spores are trapped onto a polycarbonate disk (Figure 6.2), which is coated with a thin layer of Vaseline to which the spores adhere. The manufacturer supplied Vaseline in a pressure can, which they recommended be used to apply a light coating of Vaseline onto the disk. However, this coated surface was found to be too coarse under high magnification and interfered with the identification of fungal spores. The method of application was altered to give a more uniform Vaseline coating. Vaseline was thinned in hexane (1:1 ratio) and then painted onto the disk with a brush. A hot-airgun

was then used to melt the Vaseline carefully to ensure a smooth surface, allowing easier observation of small indistinct spores at high magnification. The disk has three-hour divisions marked into the plastic and has an eight-day spore-trapping capacity. The disk is removed from the trap and placed onto a microscope adaptor. The disk surface can then be rotated past the microscope objective for observation of any trapped spores.

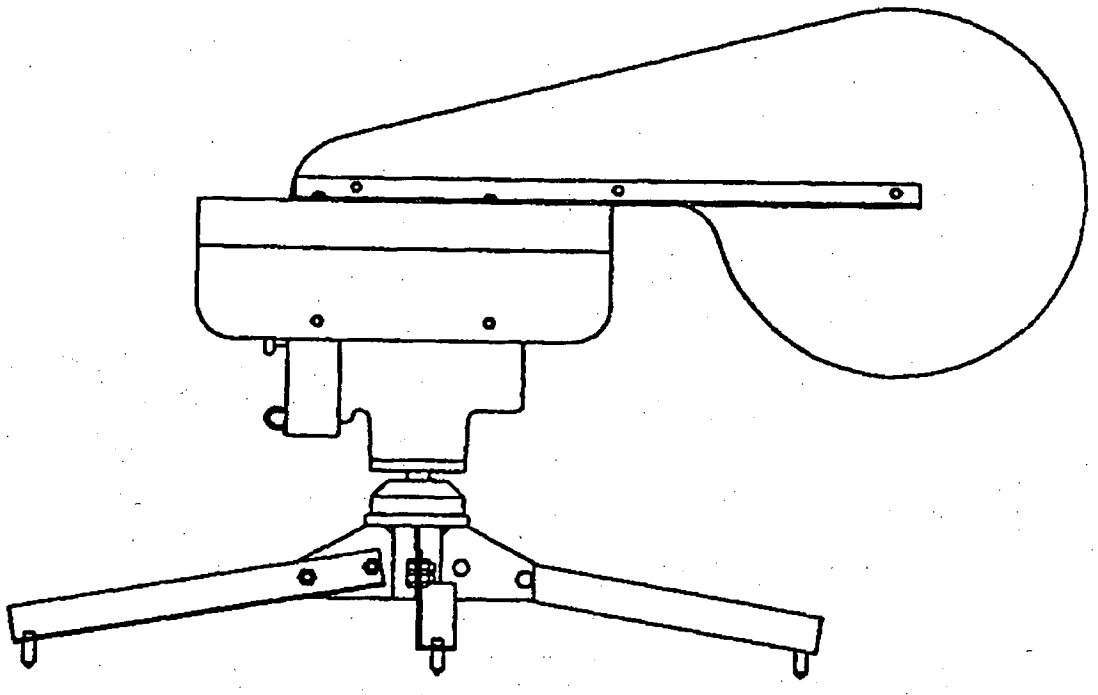

Figure 6.1: Diagram of the Quest spore trap (taken from the Quest spore trap manual).

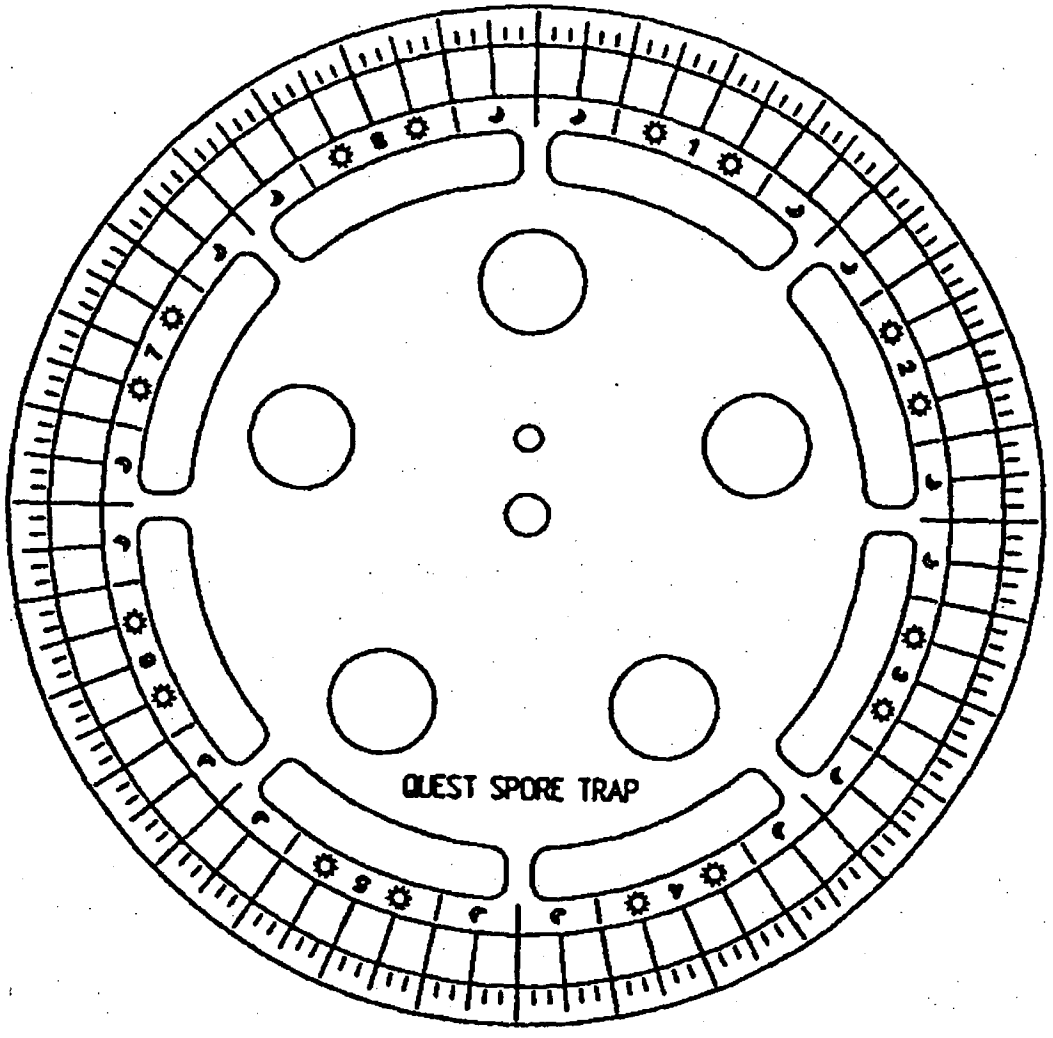

Figure 6.2: Quest spore trap disk (reproduced from the Quest spore trap manual). The band used to count spores is 10 mm wide

Calculation for volume of air sampled

The volume of air sampled by the trap was estimated to quantify the number of spores in the air at particular sampling times. The following calculation assumed that all of the 20 L of air that is sampled per minute impacted onto the trap area and that the impact efficiency for the spores of interest was 100%.

The calculation was the best estimate of the amount of air sampled.

Internal diameter of the sampling band = 163 mm. Internal radius = 81.5 mm

Outside diameter of the sampling band = 183 mm. Outside radius = 91.5 mm

Disk sample area $= \pi \times 91.5^2 - \pi \times 81.5^2$

$= 5434.95 \text{ mm}^2$

Sample area of squares (64 in total) = 84.92 mm^2 per 3 hr

At 20 L of air sampled per minute

Air sampled per 3-hour square = 180 min x 20 L

= 3600 L/3-hour square or 1200 L per hour

Microscope objective area

20X objective, diameter of field of view = 1 mm, area = 0.78 mm^2

$\therefore$ 20X objective = 33.07 L of air sampled

In Experiment 1, 10 fields of view were examined at 200X magnification per 3 hour segment of the spore trap. This equated to 330.7 L of air for that 3-hour time period, i.e., 330.7 L out of a potential 3600 L sampled over the 3-hour period, which equated to 9.2% of the disk trap surface area. For analysis purposes the number of spores trapped was converted to spores per cubic meter.

6.2.3 Spore staining for identification

A method to assist in the identification of *B. allii* spores was trialed. It was envisaged that this staining method could assist in identification of *B. allii* conidia from spores of other *Botrytis* spp. and in differentiation from background material, including other fungal spores, pollen and dust. The nuclei staining method was modified from Shirane *et al.* (1989) and Dhingra and Sinclair (1985) for use on the spore trap disk (Appendix 12.6). For training and experimental purposes, large numbers of *B. allii* spores were trapped onto the disk by disturbing *B. allii* conidiophores on an infected onion in front of the aperture of the operating spore trap. Spores of *B. cinerea* from a sporulating Pectin Agar culture were also trapped and stained to assess the visual difference between spores of the two species. The disk was then stained following the methods listed in Appendix 12.6. One hundred conidia of *B. allii* and *B. cinerea* each were examined.

6.2.4 Weather station

Air temperature, relative humidity, leaf wetness and rainfall were measured using a data logger (WatchDog model 450 data logger manufactured by Spectrum Technologies, Inc, Illinois). The data logger had internal temperature and humidity sensors and an external leaf wetness sensor (cat #3666, gold-plated) and a rain gauge (cat #36651 – capable of 1/100th-inch increments of measurements with +/-4% accuracy). Leaf wetness was recorded on a scale of 0 (dry) to 14 (wet).

6.2.5 Experiment 1 – Volumetric spore trapping from an onion cull pile

6.2.5.1 Inoculum source

Onions bulbs were inoculated by injecting a suspension of *B. allii* isolate C3 into the neck of approximately 50 onions (as described in Appendix 12.3). The bulbs were then cut in half 1-week later and left to sit on the laboratory bench for 2 weeks to sporulate. Onions were placed around the trap at 3 pm on the 16/8/00 (Figure 6.3). The trap was started a few hours later so that any spores initially released as a result of placement of the bulbs did not affect the results.

A sprinkler was used to simulate precipitation on 20/8/00 at around 12 pm for 30 minutes.

Figure 6.3: Volumetric spore trap and weather station, with *B. allii* inoculum in the form of onion bulb halves bearing conidiophores.

6.2.5.2 Quest volumetric spore trap

The trap was operated from 6 pm on the 16/8/00 to 12 pm on the 24/8/00. The weather data logger was also run for the same period. The spore trap disk was stored in the refrigerator at 7°C until it could be examined. The disk was assessed within 2 weeks of trapping without any observable deterioration of spores. No spore staining was used in this experiment. Assessment of the trap disk was made for each 3-hour segment, by taking a diagonal transect with the 20 X microscope objective, which consisted of 10 fields of view along the transect.

6.2.6 Experiment 2 – volumetric, filter and rain spore traps on a cull pile

6.2.6.1 Preliminary filter trap experiments

The filter traps consisted of a battery-powered aquarium air pump and a Millipore filter and filter holder (Figure 6.4). This arrangement had been used to monitor dust levels along walking tracks in the Daintree Forest (Pia Anthony, *personal communication*, James Cook University, Townsville, Queensland, Australia). The filters used in the traps were 0.45 μm nitrocellulose Millipore-filters (25mm diameter) which were autoclaved in plastic holders before use. Air was drawn through the filters using a battery-powered mobile aquarium pump (Shiruba, Aerator K-101), in which the valves had been reversed (by rotating the valve unit 180°) so as to reverse the air flow of the pumps. The pumps had 1.5-volt motors powered by two D-size batteries (in parallel). The batteries were replaced as they ran flat over the course of the experiment.

Figure 6.4: Aquarium pump and filter trap

On the laboratory bench, 5 filter traps were placed at each of 20, 50, 70 and 100 cm distances from a pectin agar plate with a sporulating colony of *B. allii*. A fan was placed 30 cm behind the agar plate to blow spores from the plate towards the traps. The traps were run for 3 hours. The filter holders from the traps were dismantled in a laminar flow cabinet and filters were placed face-down onto Kritzman's agar (KA) in Petri plates. The surface of the filter from one trap (Trap 2) was rinsed with sterile distilled water onto a plate of KA and then the filter was also placed face down onto a separate KA plate. Plates were incubated in the dark at 20°C. After a 24-hour period, all of the filters were moved across the agar plate, to allow any colonies growing into the agar to sporulate. Fungal colonies could be seen after 1-week incubation. After 2 weeks of incubation, the *Botrytis* colonies were identified under the stereo microscope as *B. allii* or *B. cinerea*.

Volume of air sampled

The volume of air moved through the aquarium pumps was determined using an inverted 1 L conical flask that was filled with water in a sink. Air from the pump was passed into the water in the flask, and the flask was lifted out of the water so that the water level inside the flask matched that outside the flask (to maintain atmospheric pressure inside the flask). The pump moved 1 L of air in 1 minute and 10 seconds. Repeating the experiment with a filter in place and sucking the air out of the inverted conical flask, it took 20 seconds to move 0.2 L of air. This only gave an estimate of the actual volume of air the pumps drew through the traps because of extra resistance in air flow as the filter collected material and as the battery voltage fell over the course of the trapping period. However, this indicated that the pumps were sampling approximately 1 L per minute or 1,440 L per 24 hours.

There may also have been variation in efficiency between pumps, and with hours of operation. Therefore, the pumps could only be used qualitatively to determine the presence or absence of *B allii* in the air sampled.

6.2.6.2 Quest volumetric spore trap

Inoculum of *B. allii* was produced on 50 onion bulbs as previously described (Section 12.3). Onion bulbs were cut in half and placed outside on grass in a circle around the Quest trap at 3 pm on 4/10/00. The Quest trap was operated from 5 pm on 4/10/00 for the duration of the experiment (6 days). A sprinkler was used to give an artificial precipitation event on the 6/10/00 from 8.30 am-9.30 am and from 10.10 pm – 11 pm, with 2 mm and 4 mm applied during the precipitation events, respectively.

For this and subsequent experiments, the spore trap arrangement was improved by increasing the number of divisions on the trap disk. The trap disk was originally marked with 3-hour divisions. To improve the relationship between time and spore counts, 1-hour divisions were marked on the disk using a scalpel. Spore counts were performed on an hourly basis over the trapping period, with six fields of view at 200X magnification per 1-hour division. This equated to 198.4 L of trapped air measured microscopically relative to the 1,200 L of air that was trapped for that 1-hour period. This measured area was equivalent to 16.5% of the disk trap surface, a higher level than in the first trapping experiment.

6.2.6.3 Filter trapping

The filter traps were set up 20-30 cm above the ground, 30 cm away from the spore source, with the filter trap inlets pointing towards the ground to prevent rain from entering them directly. The traps were run for 24-hour periods from 6 pm to 6 pm the following day. The batteries were changed in the traps as they ran flat. The D-sized batteries generally ran for 2 days before needing replacement.

6.2.6.4 Rain collection traps

Tin cans were used to trap water and spores during a rain event. The cans (7-cm-diameter) were placed in a straight line on the ground for a 26-hour period (9 am on 7/10/00 to 11.30 am on 8/10/00), at 0.00, 0.15, 0.35, 0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 7.00 and 10.00 meters from the inoculum source. The rain caught per can (from 5.0 to

9.5 ml per can) was transferred to individual 15 ml centrifuge tubes and shaken vigorously with a drop of Tween 80, to ensure spores were in suspension. The tubes were centrifuged at 3000 rpm for 20 minutes (Evenhuis *et al.*, 1997). The supernatant was poured off and the pellet resuspended in 2 ml of dH_2O. An aliquot (1 ml) of the suspension was spread onto Kritzman's agar in Petri plates with a bent glass rod, and the plates allowed to dry in the laminar flow cabinet. This gave a ½ dilution of the spores from the cans. A further aliquot (0.1 ml) of suspension was spread onto KA in Petri plates with a bent glass rod to give a 1/20 dilution of the spores from the cans. Plates were covered and incubated for a week and assessed for growth of *B. allii* with the aid of a dissecting microscope. To analyse the spore dispersal gradient, two empirical models of gradient analysis were fitted to the data set, the power law model and exponential model. Full explanation of the data analysis is given in Chapter 7.

6.2.7 Experiment 3 – Volumetric and filter spore traps and trap plants in an onion crop

This experiment was conducted as part of Field Trial 2 (Chapter 8) which compared the effect of a normal irrigation regime compared to a high irrigation regime on an epiphytotic of *B. allii*. Sprinklers in the high irrigation area were run for 10 minutes at 8 pm on the 20th, 21st, 22nd and 24th of February 2001. Various methods of trapping spores (below) were utilised.

6.2.7.1 Quest volumetric spore trapping

The Quest volumetric spore trap was started on the 15/2/01 at 11 am, and was run for the 2-week duration of this third spore trapping experiment. The trap was located in Field Trial 2, between Beds 1 and 2 at the intersection of Quadrats 5 and 6 in the high irrigation treatment area (Chapter 8 Section 8.2.3). In this experiment, the inoculum for the trap originated from the crop. The trap was checked every 2 days for timing and adjusted as necessary. The disk was changed at 8 am on the 22/2/01, and stored in the refrigerator at 7°C until it could be examined (within a week). The disk was examined as previously described. However, on this occasion the disk was measured twice (referred to as Volumetric Counts 1 and 2) to determine how much variability existed in the disk assessment.

6.2.7.2 Filter traps

To reduce costs and increase the reliability of the power source for the filter traps, the D-cells that were used to power the filter traps in Experiment 2 were replaced with car batteries. One 39k-ohm ceramic (PW10) resistor was used to regulate the current of each filter trap. Similar current levels and motor speeds to the D-cell batteries were attained using the car batteries with resistors. Each car battery was used to power two filter traps. Filter trapping was started at 7 pm on the 15/2/01 and the filters and batteries were changed every 24-hour period, for the 2-week trapping period. The filter traps were placed in the middle of the infection bed, between quadrats 5 and 6 in the high irrigation treatment area, close to the Quest volumetric spore trap (Chapter 8, Section 8.2.3).

6.2.7.3 Trap plants

Onion trap plants were sown 10 seeds per pot on the 7/11/00 in a peat/sand potting mix in 14-cm-diameter pots. The plants were thinned to 3 plants per pot. Plants were maintained in a greenhouse. The pots were irrigated once a day at 6 am for 1 minute with a micro-irrigation system. Soluble fertiliser (Yates® Thrive, N:P:K = 27:6:9 with micronutrients) was applied on the 13/12/00 and the 27/12/002 as per manufacturers directions. Two and a half grams of slow release Osmocote® fertiliser granules (N:P:K = 15:4:10 with micronutrients) were applied to each pot on the 3/1/00. Plants were also sprayed with a pyrethrum-based insecticide to control thrips on the 3/1/01 with a follow-up application on the 8/1/01.

Trap plants were placed in the field of Field Trial 2 (Chapter 8) for 24-hour periods from 7 pm for the duration of Experiment 3 (14 days). To maximise the likelihood of infection, the trap plants were spaced along the infection bed. Ten pots of trap plants were placed in the field during each 24-hour trapping period. Five pots were placed in the high irrigation treatment area, with pots placed between the quadrats 1-2, 3-4, 5-6, 7-8 and 9-10 (Chapter 8, Section 8.2.3). The same pattern was also used for the five pots that were placed in the low irrigation area. After the plants had been exposed in the field, they were taken back to the glasshouse, placed into a chamber with low humidity and were watered from the base. Low humidity was maintained to prevent spores on leaf surfaces from infecting the leaves or new infections from occurring in the greenhouse. After 12-14 days, the leaves were harvested and placed into 1-L plastic food trays lined with moistened tissue paper. The leaves were incubated for 1 week at room temperature. The number of leaves infected with *B. allii* and/or *B.*

cinerea per plant was assessed. Leaves from trap plants that had not been exposed to the trial site were also incubated as a control to check for any background level of *Botrytis* infection in the greenhouse.

6.2.8 Analysis of methods of weather and spore trap data

It was postulated that weather conditions in the day or days prior to spore release may be important determinants of spore release. Using lag periods of 1 to 5 days, an attempt was made to correlate weather data with spore trap data.

The wind speed data was collected from a station on top of a greenhouse located less than 3 km from the trial site. An average wind speed was calculated over 2-hour periods and then correlated with the sum of the spore counts for the corresponding 2-hour periods.

6.2.9 Spore survival in sunlight

A pilot investigation was made into the effect of sunlight on survival of *B. allii* conidia. Spores were brushed from an onion inoculated with *B. allii* isolate C3 (Appendix 12.9) into 6 Petri plates, the lids of which were sealed with clingwrap, and placed outside on the 10/9/99 at 12.30 pm. Half of the plates were retrieved at 2.30 pm on the same day and the rest retrieved at 2.30 pm on the 11/9/99. Upon retrieval, 2 ml of a Tween 80 suspension (one drop of Tween 80 per 20 ml of dH_20) was placed into the Petri plates, and agitated to ensure spores were in suspension. An aliquot (1 ml) of the suspension was plated onto 1.0 % water agar in Petri plates and spread with a bent glass rod. The plates were then dried (for 30 min) of free water in the laminar

flow cabinet before being covered, sealed with clingwrap and incubated at 20°C in the dark. Sunlight intensity during the period of exposure was measured at a site 50 km away (Forthside research weather station). The experiment was arranged as a completely randomised block design and analysed with ANOVA.

6.3 Results

6.3.1 Nuclei staining

After modifying the procedure of vaseline application to the spore trap disk and the staining procedure of Shirane *et al.* (1989) it was possible to stain the nuclei of conidia on the disk surface of the Quest spore trap (Figure 6.5). It was also possible to differentiate conidia of *B. allii* from those of *B. cinerea* based on the number of nuclei per conidium. Generally, conidia of *B. allii* had only one nucleus whereas *B. cinerea* conidia had 3 nuclei. Shirane *et al.* (1989) reported that *B. allii* isolates (including both AI and AII types) had 1.3-1.5 nuclei per conidium on average. *Botrytis cinerea* isolates were reported as having 4.0 to 5.1 nuclei per conidium on average. However, the number of nuclei observed in this study varied, as demonstrated in Figure 6.5. Therefore, it was considered that this technique could not be used alone to differentiate conidia of a mixed species population. In practice, it was found that the staining technique was time-consuming, both in terms of staining the disk and observing the spores, as the spores had to be observed under high magnification (1000X) with a coverslip in place. It was also a concern that spores could be dislodged from the disk surface with the various washes of solutions during the staining procedure.

Differences in shape and size of conidia between *B. allii* and *B. cinerea* were found to be sufficient for identification of single conidia to species. Therefore, the decision was made to perform spore disk assessments without the staining procedure and to rely upon the refractive characteristics of the spores (the spores were a translucent blue colour when using the fine focus on the microscope), and size and shape of the *B. allii* conidia for identification. Spores of *B. cinerea* were not as much of a problem in identifying spores of *B. allii* as the dirt and other debris that was trapped on the disk surface, which sometimes obscured spores.

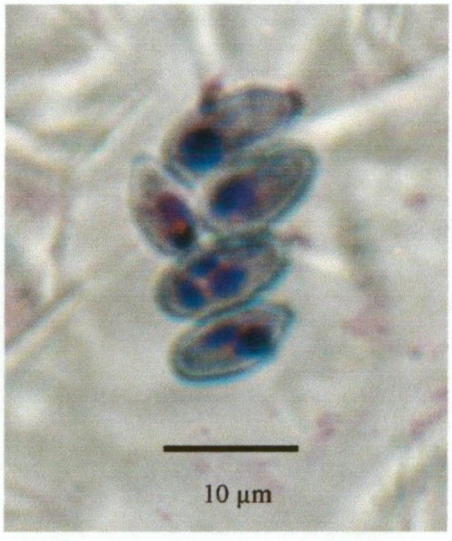

Figure 6.5: *Botrytis allii* conidia stained with Giemsa nuclear stain as observed at 1000X magnification, demonstrating the variability in the number of nuclei per conidium.

6.3.2 Preliminary spore trapping experiments with filter and agar traps

All of the filters from the filter traps tested positive for the presence of *B. allii* (Table 6.1) when exposed to windblown inoculum on the laboratory bench. This demonstrated that the traps were capable of trapping spores of *B. allii* from the air. The experiment also demonstrated that washing spores from a millipore filter onto a

plate of Kritzman's agar was a successful method for detection of *B. allii*. No statistical analysis was performed on this data set.

Table 6.1: *Botrytis allii* detected on filter traps on a laboratory bench over a three-hour period.

Trap	Distance from Petri plate	*B. allii* detected (+) or not detected (-), after plating filters onto Kritzman's agar
1	20 cm – rinsed filter surface	+
2	20 cm – filter placed on Kritzman's Agar (KA)	+
3	50 cm – "	+
4	70 cm – "	+
5	100 cm – "	+

The experiment was repeated on the laboratory bench, including the use of Kritzman's Agar (KA) plates placed out with the filter traps. The numbers of *B. allii* colonies found per plate decreased with distance from the inoculum source (Table 6.2). Spores were found at all distances monitored by trapping directly onto KA in Petri plates. However, *B. allii* was only detected on 4 of 7 filter traps (Table 6.2).

Table 6.2: *Botrytis allii* detected on filter traps or Kritzman's agar on a laboratory bench over a three-hour period.

Distance from source of inoculum (cm)	*No. of filter traps with B. allii / total no. of traps*	*Number of B. allii colonies/ KA plate*	
		Plate 1	*Plate 2*
10	1/2	18	-
30	3/3	11	15
60	0/1	9	6
100	0/1	4	4

Petri plates were also trialed outside but were found not to be suitable for trapping spores for any great length of time, as the plates tended to dry very quickly even when shielded from direct sunlight. The plates would be of use when trapping spores for very short periods of time or during damp weather. No statistical analysis was performed on this data set.

6.3.3 Effects of humidity, free water and spore concentration on spore germination

After 3.5 hours incubation on water agar plates germinating spores were not observed at either spore concentration evaluated (Figure 6.6). By 16.5 hours, 54% and 68% germination was observed for the 8 x 10^5 and 3.2 x 10^5 spores suspensions, respectively. By 23 hours, 65% and 75% germination was observed, respectively. At each sample time, the differences in germination between spore concentrations were not significant. However, if a greater range in spore concentration had been examined perhaps a significant difference may have been found.

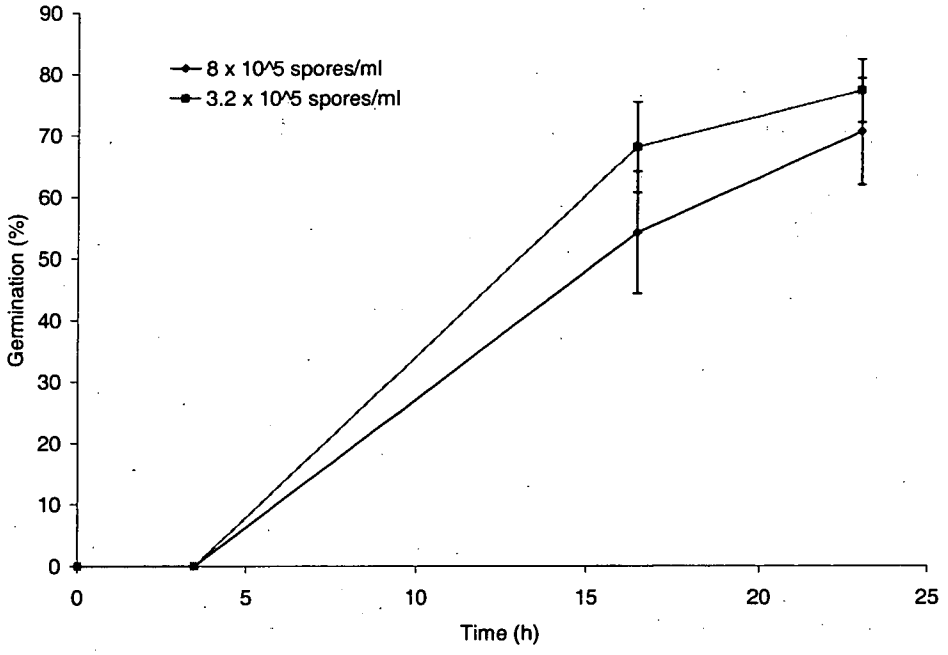

Figure 6.6: Time required for spores of *B. allii* to germinate on 0.5% water agar, at 25°C in the dark, at two different spore concentrations. Bars represent standard deviations of the 6 replicates per mean.

The experiments also demonstrated an apparent need for free water for germination. Spores did not germinate on the glass slide surface even after 48 hours at very high humidity. Saturation of the air was reached in the experiment carried out using food trays, with the formation of water droplets on the lower surface of the glass slides. However, the spores did germinate on water agar, which contained no stimulant apart from free water. Based on these experiments, it appears that free water is required for the germination of *B. allii* spores.

6.3.4 Experiment 1 – Volumetric spore trapping from an onion cull pile

Three main peaks of spore activity were noted in this experiment (Figure 6.7). The first peak appeared to correspond with the irrigation event on 19/8/00 and the final

peak with a period of rain. Attenuation of the first peak suggested a majority of the conidia were dislodged by irrigation/rainfall, but additional conidia were released over a period of time afterward, perhaps as a result of wind or subsequent rainfall. There seemed to be no relationship between spore release and RH (figure 6.8), leaf wetness (Figure 6.9) or air temperature (Figure 6.10). The spore peaks may also have resulted from a combination of favourable conditions for spore production prior to spore release events. Perhaps water droplets (from the irrigation event) mechanically dislodged conidia produced following favourable conditions for sporulation in the laboratory. The two subsequent peaks may be a result of favourable conditions leading to the build-up of secondary flushes of conidia, released by further precipitation and/or wind events.

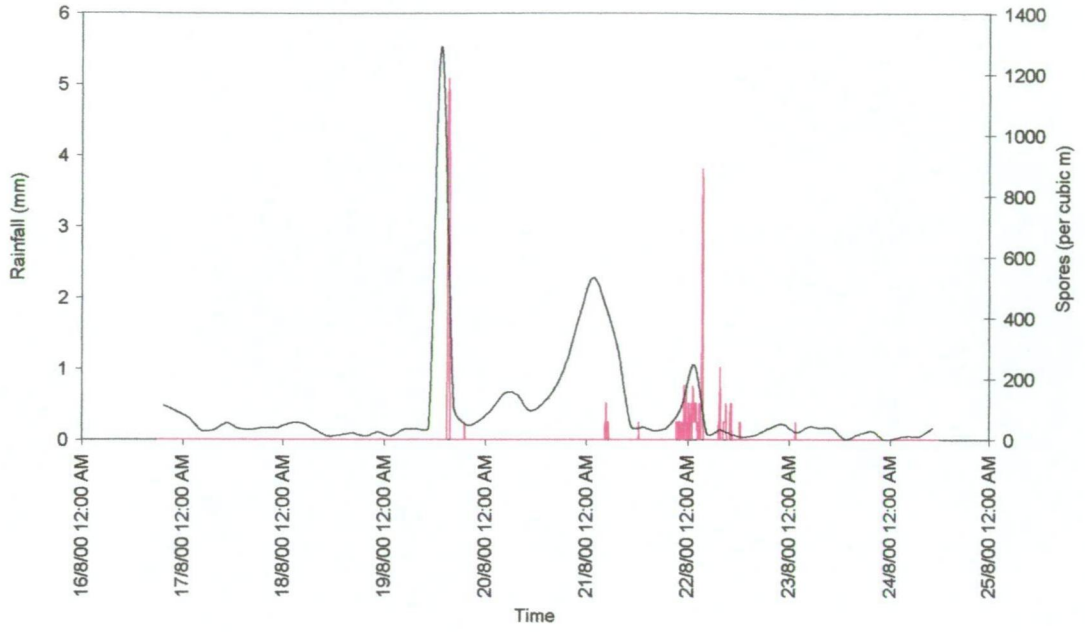

Figure 6.7: Rainfall (—) and spores of *B. allii* (—) trapped in the air over time. The onions were given a precipitation event on 19/8/00, which is seen as the first peak on the rainfall graph, the following peaks were from rainfall events (Experiment 1).

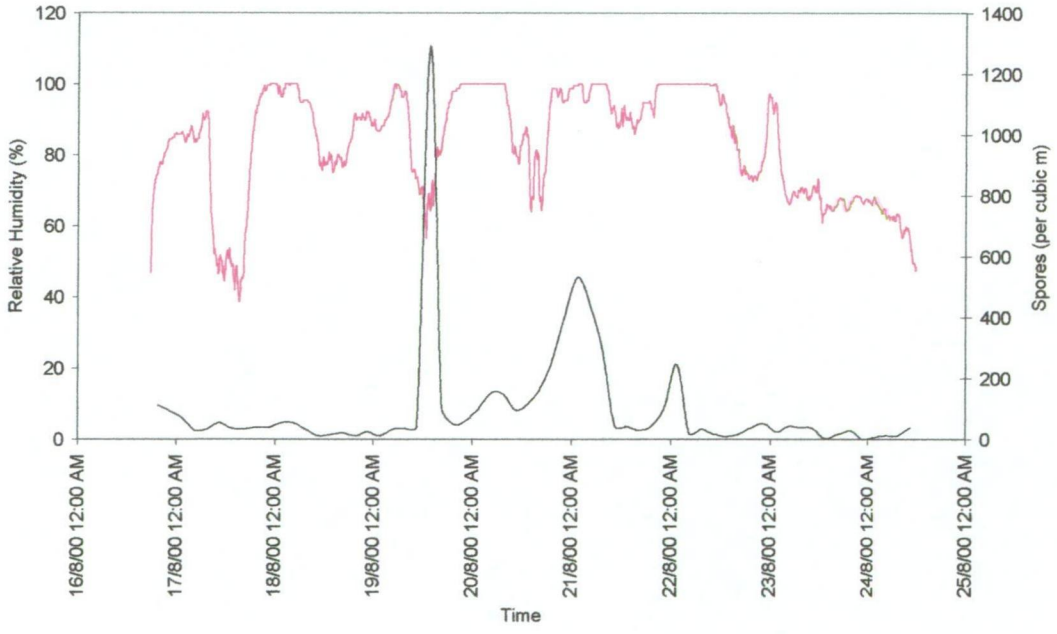

Figure 6.8: Relative humidity (—) and spores of *B. allii* (—) trapped in the air over time (Experiment 1).

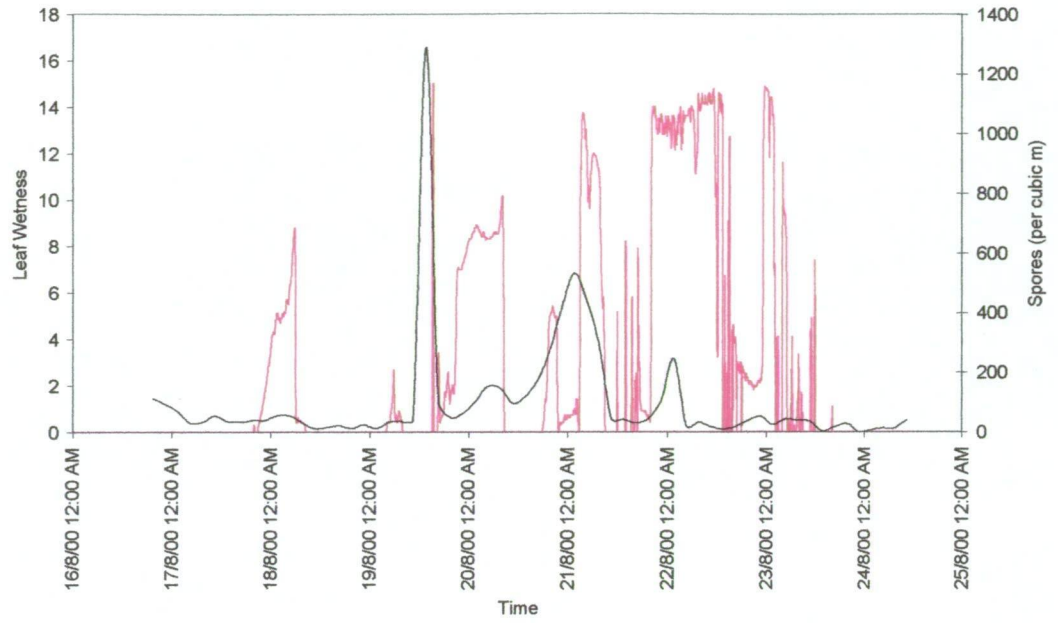

Figure 6.9: Leaf wetness (—) and spores of *B. allii* (—) trapped in the air over time (Experiment 1).

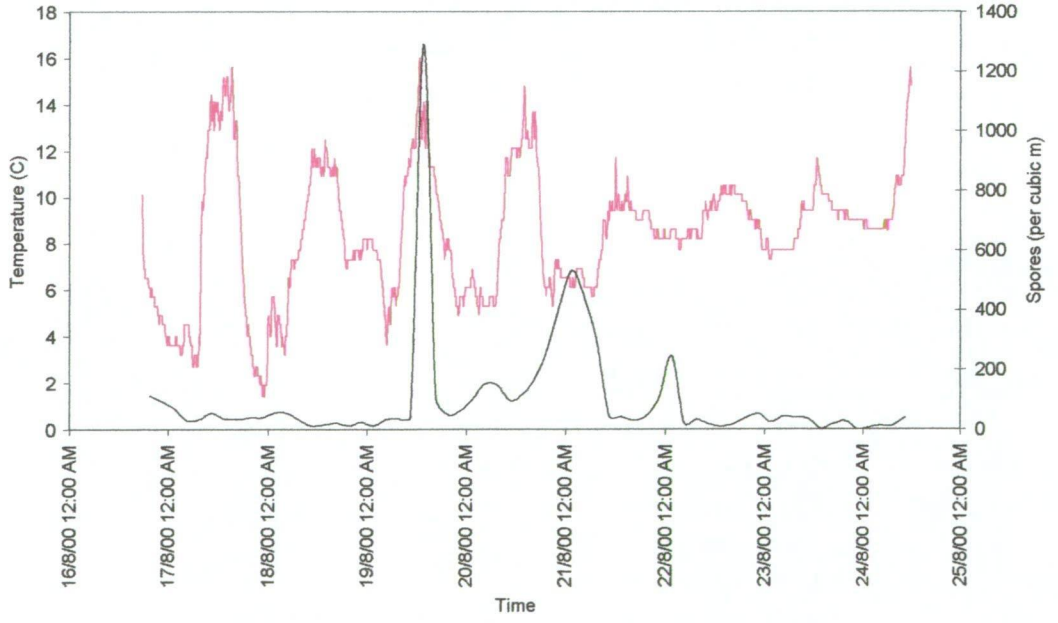

Figure 6.10: Air temperature (—) and spores of *B. allii* (—) trapped in the air over time (Experiment 1).

6.3.5 Experiment 2 – Volumetric spore trapping, filters and rains traps from a cull pile

6.3.5.1 Quest volumetric spore trapping

The sprinkler events can be seen as the first two peaks in the rainfall graph (Figure 6.11). From the information on rainfall from the data logger, rainfall was recorded for a period of 15-30 minutes after the rainfall event had ceased. This lag was due to the nature of the water gauge, in which water collected in a tube and ran through a wick which produced droplets that fell between sensors.

In Experiment 2, rainfall again appeared to be associated with the release of conidia (Figure 6.11). A small peak in spore numbers trapped occurred before the main spore release event. Although no rain was recorded at this time, leaf wetness had climbed sharply (Figure 6.12), indicating a rain shower may have occurred that did not register on the rain gauge as the volume was too low. However, the main peak in spore release corresponded with the irrigation event. No major peaks were recorded after this time even though there were subsequent rainfall events (Figure 6.11). The first major peak in spore activity also appeared to coincide with a time of declining RH (Figure 6.13) and increasing temperature (Figure 6.14).

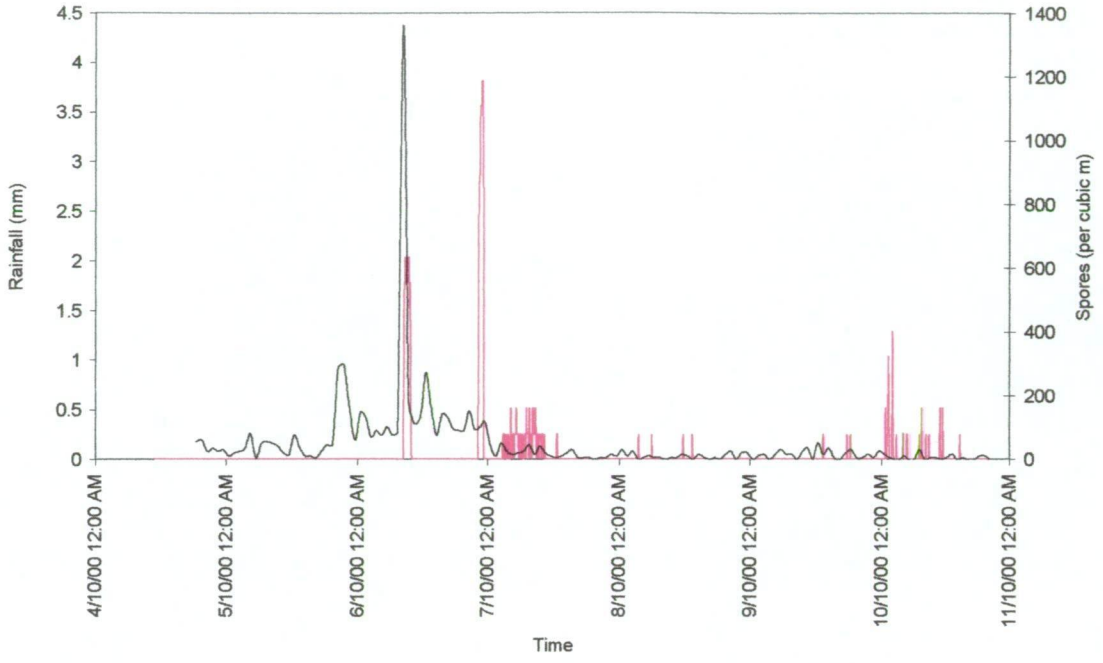

Figure 6.11 Rainfall (—) and spores of *B. allii* (—) trapped in the air over time. The first two rain events recorded are from use of a sprinkler (Experiment 2).

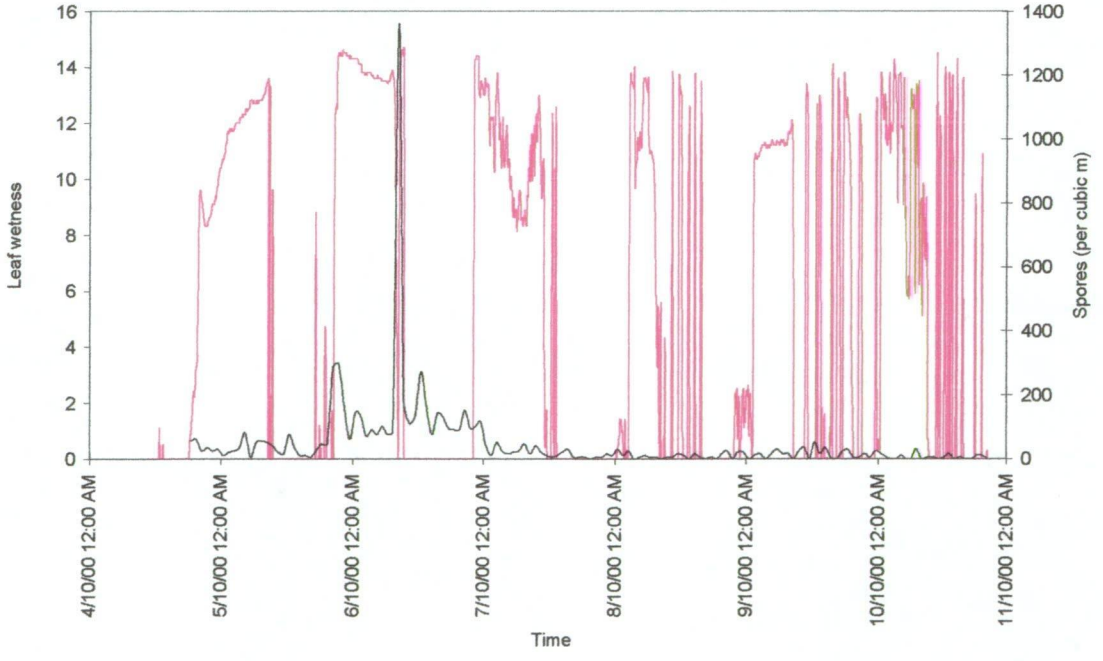

Figure 6.12: Leaf wetness (—) and spores of *B. allii* (—) trapped in the air (Experiment 2)

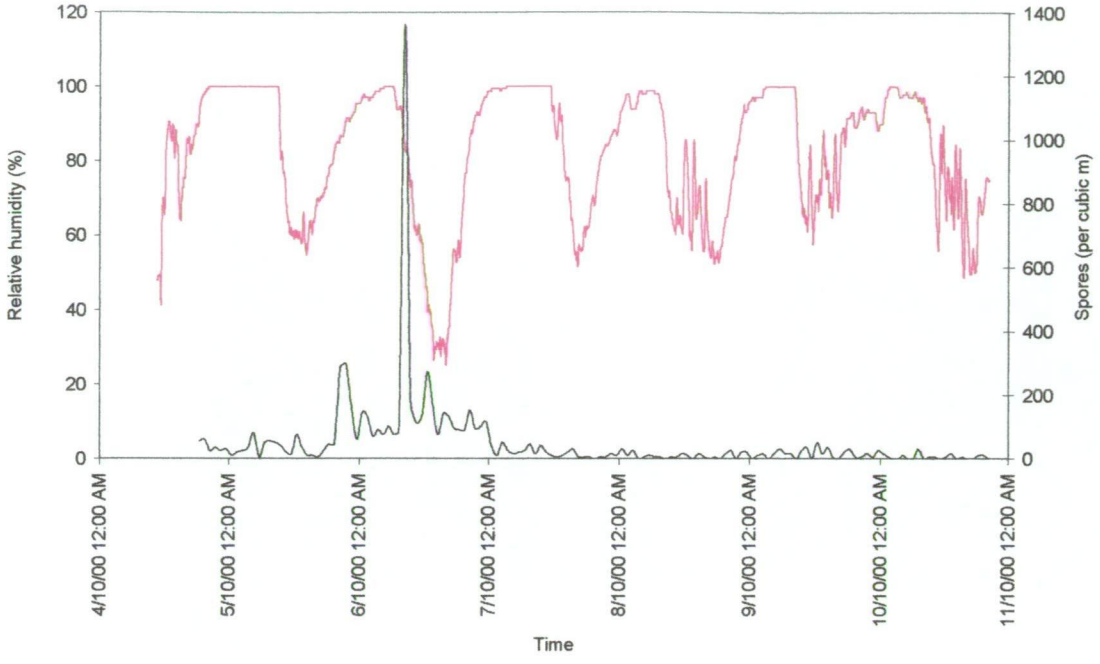

Figure 6.13 Relative humidity (—) and spores of *B. allii* (—) trapped in the air (Experiment 2)

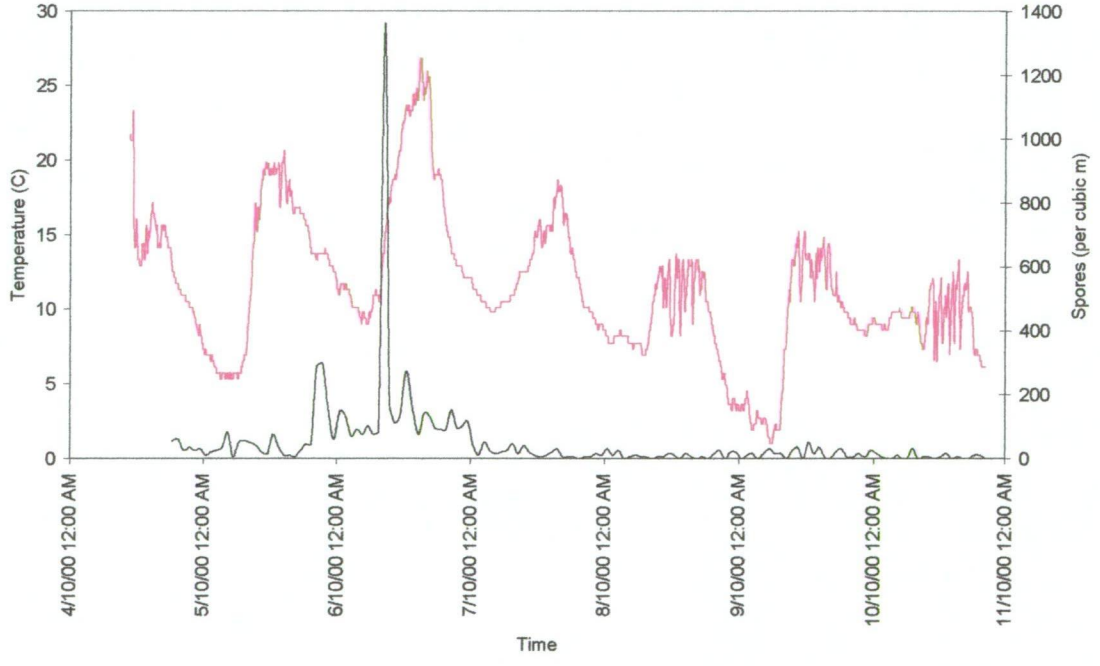

Figure 6.14: Air temperature (—) and spores of *B. allii* (—) trapped in the air (Experiment 2)

6.3.5.2 Filter traps

The filter traps demonstrated that spores were released during each 24-hour period monitored (Table 6.3). This supports the Quest volumetric trap observations of spores being present in the air most of the time. Some of the filter traps failed during the trapping periods due to flat batteries or mechanical problems with the motors, these are not shown in Table 6.3.

Table 6.3: Results of 24-hour periods of filter trapping for *B. allii* in experiment 2.

Day	# of filter traps that tested positive for *B. allii*
1	5/5
2	4/5
3	5/5
4	5/5
5	4/5
6	4/5
7	5/5
8	5/5

6.3.5.3 Rain trapped spores

The ½-dilution of the centrifuged spore suspension from the cans used to catch rainfall precipitation, and plated onto KA produced a high number of CFUs, too many for counting individual *Botrytis* colonies. The 1/20 dilution plates gave suitable numbers of *Botrytis* colonies for counting. Data is presented as CFU per can in Table 6.4.

Table 6.4: *Botrytis allii* and contaminants obtained from rain traps for a trapping period of 26 hours during a rainfall event (Experiment 2).

	Distance from inoculum source (m)										
	0	0.15	0.35	0.5	1	2	3	4	5	7	10
	Volume trapped (ml)										
	9.5	8.5	8.5	9.0	5.0	6.0	9.5	6.5	5.5	5.0	6.5
No. *Botrytis* colonies on KA	+[1]	10	2	2	0	1	0	0	0	0	0
Total no. fungal colonies	#[2]	54	44	36	10	13	37	31	7	14	1
No. bacterial colonies on KA	#[2]	2	9	10	8	10	8	4	83	5	114
B. allii CFU per can	#[2]	200	40	40	0	20	0	0	0	0	0

+[1] indicates *Botrytis* colonies present. #[2] too numerous to count.

The amount of rain retrieved from the cans ranged from 5-9.5 ml. According to the weather station data, 3.5 mm of rain fell over the rain-trapping period. Therefore, the following amount of rainwater was trapped;

$$V = \pi r^2 \times d$$

$$= \pi \times (3.5\ cm)^2 \times 0.35\ cm$$

$$= 13.5\ cm^3 \text{ (or ml) per can}$$

As there were no spores detected in the last 4 data points, they were removed from the analysis. The power law model fitted the spore gradient slightly better than the exponential model with a high R^2 of 0.98 (Table 6.5).

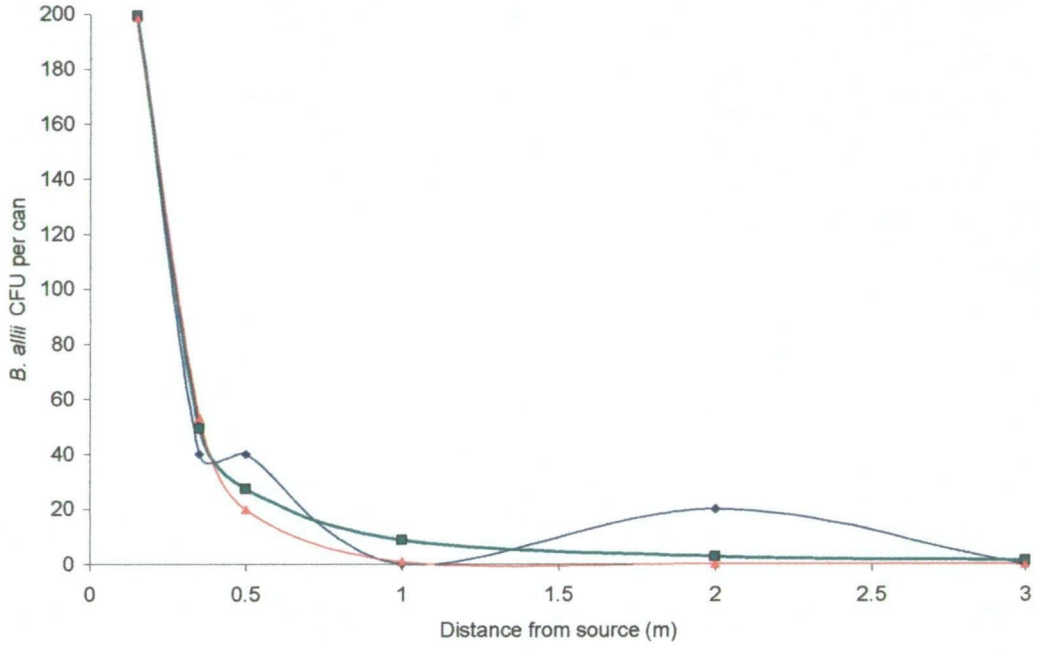

Figure 6.15: Power law model (—) and exponential model (—) fitted to the actual (—) data set of *B. allii* spores caught in rain traps at increasing distances from a cull pile.

Table 6.5: Regression results of the power and exponential model of *B. allii* spores caught in rain traps at increasing distances from a cull pile.

	Power law model = A x distance ^{B}			Exponential model = A x exp (-B x distance)		
Data set	R^2	A	B	R^2	A	B
CFU in cans	0.98	8.71	-1.65	0.96	773.2	-22.38

Power law model: $Y = 8.7375 \times^{-1.6483}$

Exponential model: $Y = 533.3679 X \exp(-6.5896x)$

Where Y = Number of CFU per can and x = distance from inoculum source.

It was estimated that a total of 302 *B. allii* spores per cubic metre were released into the air over the rain trapping period, as detected by the Quest volumetric spore trap.

6.3.6 Experiment 3 – Volumetric and filter spore traps and trap plants in an onion crop

6.3.6.1 Volumetric trap data

At the time the Quest trap was placed out in the field trial, conidiophores of *B. allii* were observed on the necks and foliage of some onion plants. Spore release in the field trial appeared to be less dependent on rainfall events than the spore release monitored from the cull piles. For example, peaks of spore release were detected in the absence of rainfall events (Figure 6.16). The number of spores detected was also much less, up to 360 spores per cubic meter than for the peaks in spore release that were detected from the cull piles which ranged up to 1300 spores per cubic meter in both Experiment 1 and 2. Peaks in the plot of leaf wetness (Figure 6.17) did coincide with the main peaks in spores trapped, however other peaks in leaf wetness were not associated with little or no spores trapped. Likewise, sharp drops and increases in RH (Figure 6.18) appeared to coincide with the main spore trapping periods, however, many other periods of changing RH were not associated with spores trapped. No pattern was apparent for the plot of temperature (Figure 6.19) against spores per cubic meter.

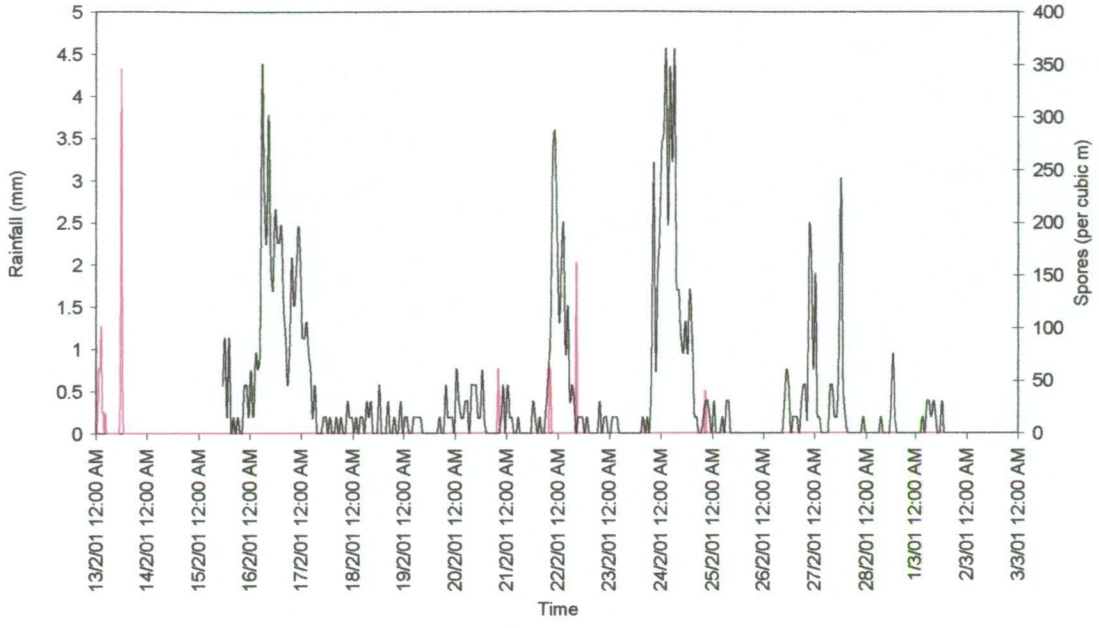

Figure 6.16: Rainfall (—) and spores of *B. allii* (—) trapped in air over time (Experiment 3).

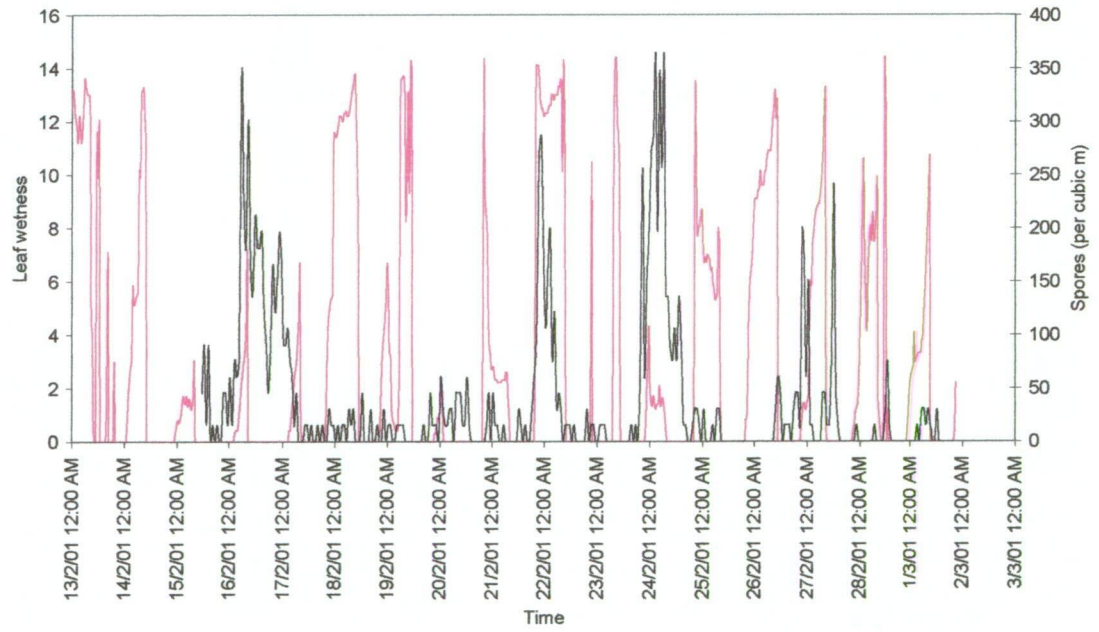

Figure 6.17: Leaf wetness (—) and spores of *B. allii* (—) trapped in air (Experiment 3).

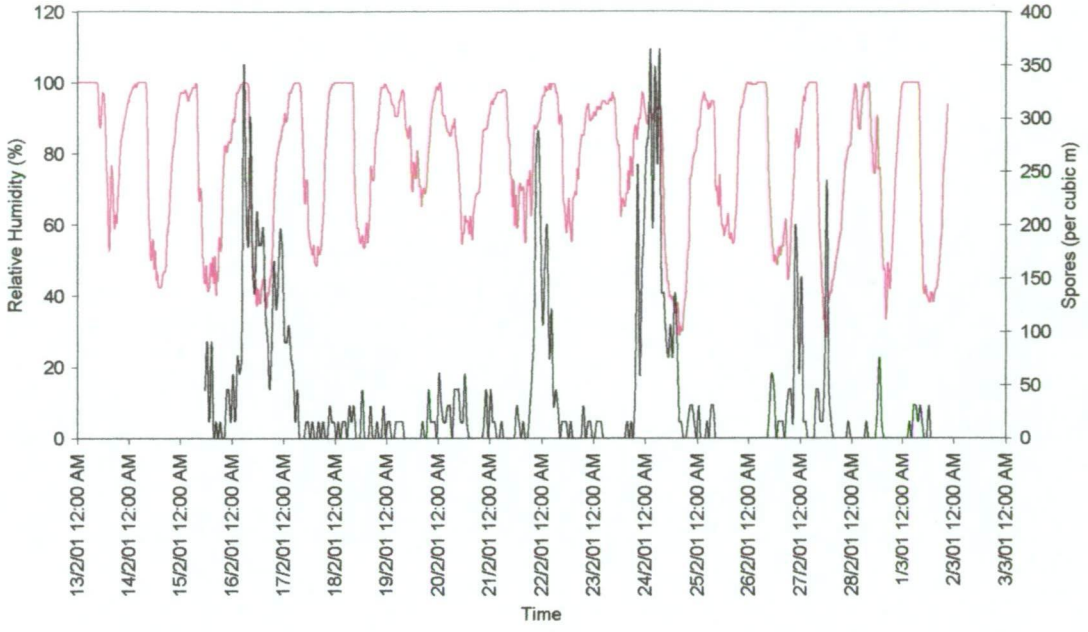

Figure 6.18: Relative humidity (—) and spores of *B. allii* (—) trapped in air (Experiment 3).

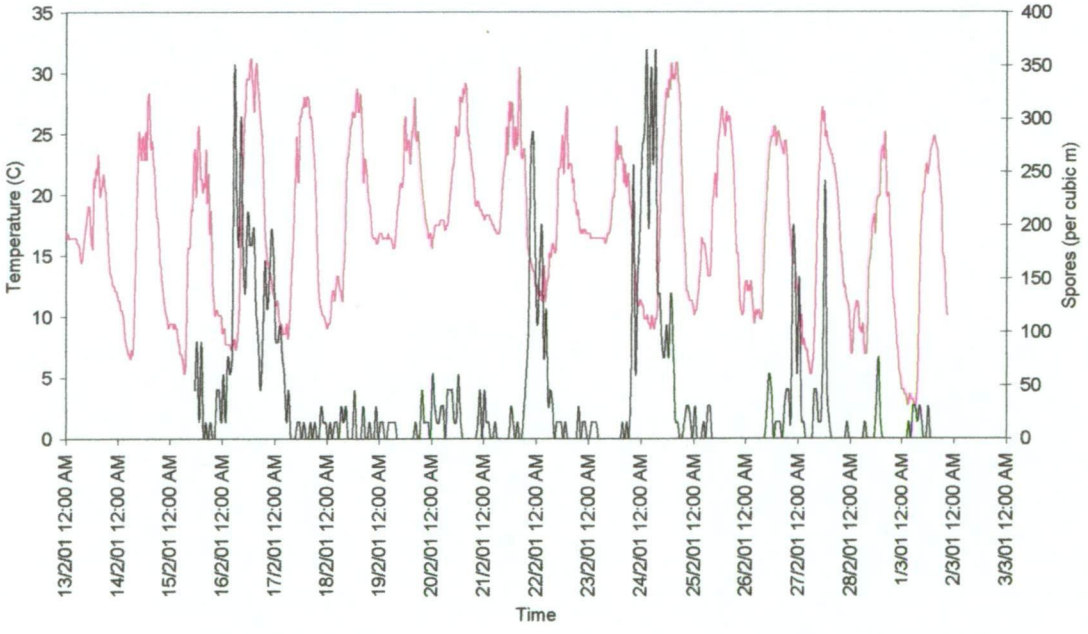

Figure 6.19: Temperature (—) and spores of *B. allii* (—) trapped in air (Experiment 3).

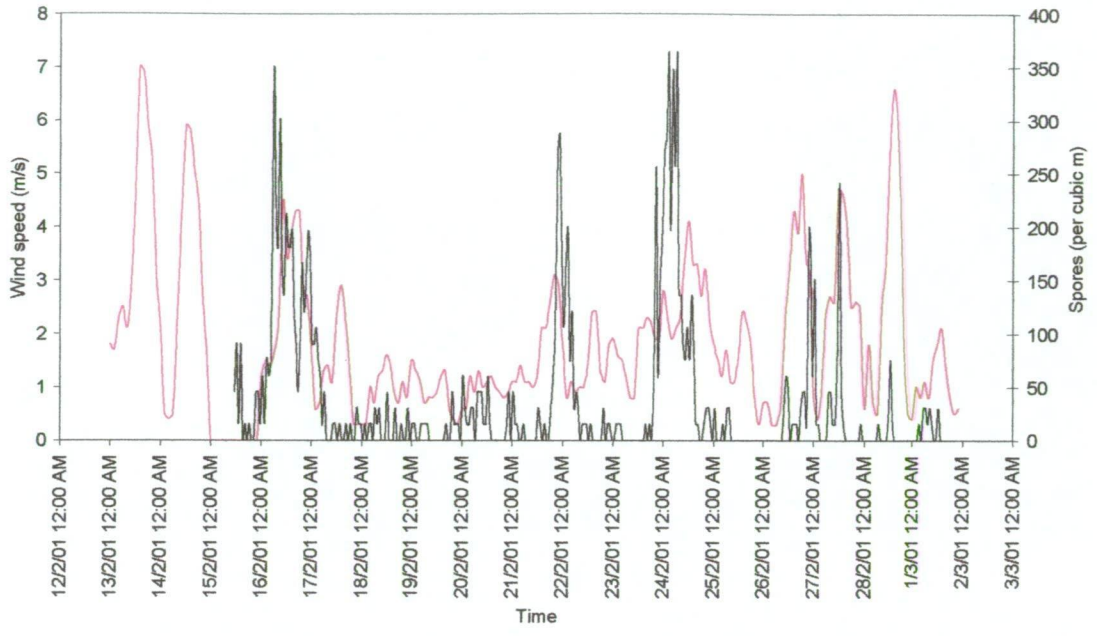

Figure 6.20: Wind speed (recorded from glasshouse station) (—) and the number of spores of *B. allii* trapped per cubic metre (—) (Experiment 3).

Peaks of wind speed appeared to be associated with peaks in numbers of spores trapped (Figure 6.20).

Analysis of weather and Quest volumetric spore trap data

The variables that appeared to be most strongly correlated with spore trap data (Appendix 12.12) were investigated in a multiple regression analysis. The regression analysis, using parameters selected from the correlation analysis, gave the following relationship:

$^{\#}Spores/m^3 = -36.1 + 3.55\ Temp\text{-}5 + 1.738\ lfwet\text{-}3 + 134.6\ rfall\text{-}3 + 29.7\ rfall\text{-}5$

$^{\#}$Temp = temperature, lfwet = leaf wetness, rfall = rainfall. Numbers indicated are the number of days lag period between the weather parameter and volumetric spore count.

The variance in the data accounted for by this regression was 24.8% and the standard error of observations was 60.0.

6.3.6.2 Filter trap and trap plant data

Botrytis allii was detected on trap plants over the trial period in both the high and low irrigation treatments (Figure 6.21). The number of infected trap plants in the high and low irrigation treatments was similar for most 24-hour periods. However, in the high irrigation treatment a higher number of infected plants was recorded over the whole trapping period, possibly due to conditions which were more favourable for spore production and release than in the low irrigation section.

Figure 6.22 demonstrates the relationship between the number of filter traps (out of a total of 4) on which *B. allii* was detected, the number of infected trap plants and the number of spores trapped in the high irrigation area. The correlation matrix (Table 6.6) demonstrates the statistical relationships among the variables.

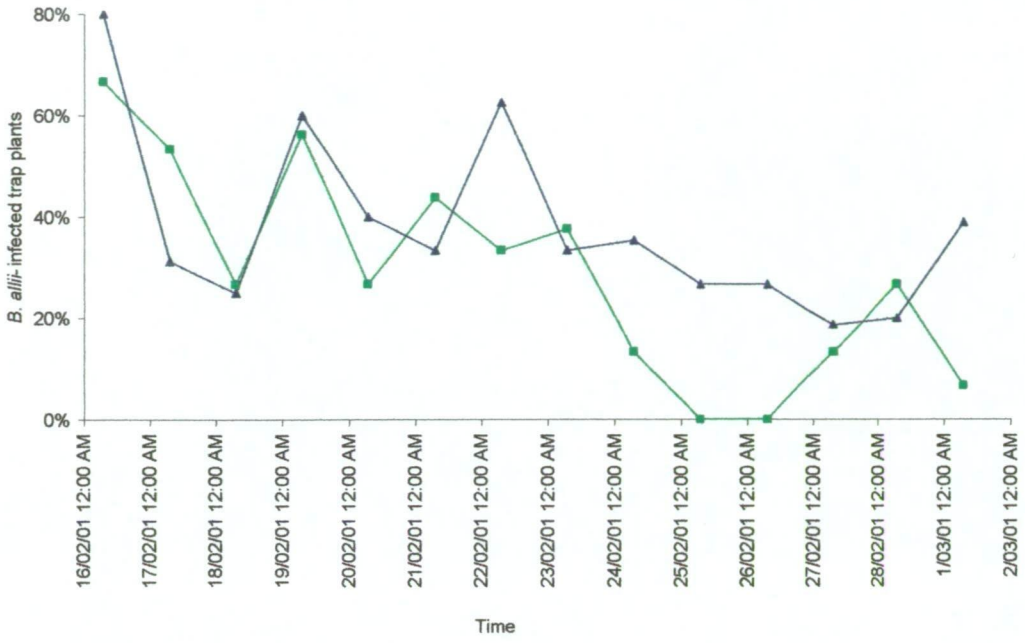

Figure 6.21: Percentage of trap plants that tested positive for *B. allii*, out of 15 trap plants per sampling period in both high (—) and low (—) irrigation treatments.

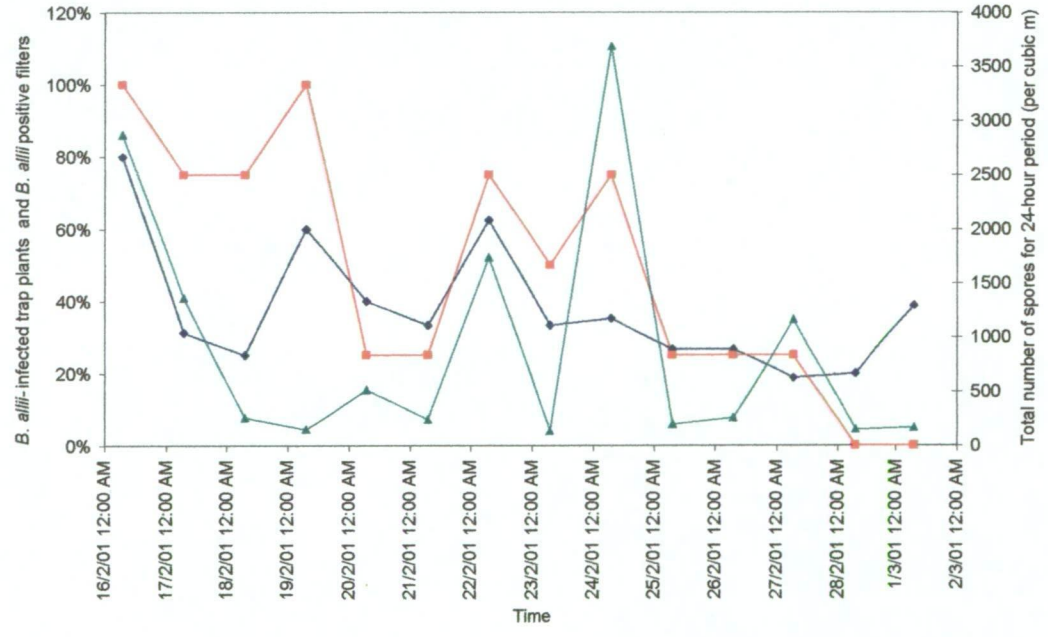

Figure 6.22: Percentage filters on which *B. allii* was detected (—) and *B. allii*-infected trap plants (—) in the high irrigation treatment and the total number of spores per cubic metre (—) trapped by the Quest volumetric trap for the 24-hour period from 7 pm to 7 pm each day from 16/2/01 to 2/3/01.

There was a significant positive correlation ($P < 0.05$) between the proportion of filter traps that tested positive for *B. allii* and number of trap plants infected with *B. allii*, from both the low and high irrigation treatments (Table 6.6). There was also a significant positive correlation between the proportion of filters on which *B. allii* was found and the counts of spores on the volumetric spore trap. The proportion of *B. allii*-infected trap plants in high and low irrigation treatments was also significantly correlated. Significant negative correlations were found between the two volumetric spore counts and relative humidity. Regression models were not fitted to this data set due to the small amount of data available.

Two separate counts were performed on the Quest volumetric spore trap. A disk was read and then repeated to check for variability in counts of spores under the microscope. The two counts were highly correlated suggesting that the technique was robust (Table 6.6).

Table 6.6: Correlation matrix ($r_{(0.05,\ 12df)}$ = 0.532) of weather variables, volumetric spore counts and incidence of *B. allii* infected trap plants.

LW	1								
RH	0.450	1							
Rain	0.528	0.280	1						
Temp	-0.160	0.441	0.108	1					
Vol 1	-0.321	**-0.581**	0.151	0.028	1				
Vol 2	-0.290	**-0.615**	0.095	0.009	**0.992**	1			
Trap plants – high irrigation	-0.072	0.077	0.341	0.159	0.470	0.396	1		
Trap plants – low irrigation	-0.268	0.304	0.061	0.348	0.242	0.170	**0.633**	1	
Filter trap	0.053	0.178	0.131	0.344	**0.544**	**0.530**	**0.656**	**0.663**	1
	LW	RH	Rain	Temp	Vol 1	Vol 2	Trap plants –high irrigation	Trap plants –low irrigation	Filter trap

Significant (P < 0.05) positive and negative correlations are demonstrated in bold.

Where:

Filter trap = no. of filters on which *B. allii* detected / total of 4 filters tested (%)
LW = leaf wetness (cumulative for the 24-hour period)
RH = relative humidity (cumulative for the 24-hour period)
Rain = rainfall (cumulative for the 24-hour period)
Temp = temperature (cumulative for the 24-hour period)
Vol 1 = volumetric spore count 1
Vol 2 = volumetric spore count 2
Plant – high = high irrigation treatment - no. *B. allii*-infected plants / 15 plants sampled
Plant – low = low irrigation treatment - no. *B. allii*-infected plants / 15 plants sampled

The correlation between the average wind speed over 2-hour periods and the total spore counts for each 2-hour period (not shown in Table 6.6) was highly significant (r = 0.266, P < 0.01). The wind speed correlation was determined separate to the other parameters in the above table, as calculations of correlation were performed on a 2-hour period basis rather than a 24 hour basis.

6.3.7 Spore survival in sunlight

The recorded solar energy for the 24-hour period (9 am-9 am) on each of 10/9/00 and 11/9/99 was 12.0 and 14.4 mj/m^2. The results suggested that spores of *B. allii* are capable of surviving at least a day of exposure to sunlight on the leaf surface, and are then able to germinate when conditions are favourable (Table 6.7). The time period of 2-hours was from 12.30 pm to 2.30 pm the same day and the 26 hour period was from 12.30 pm on the 10/9/99 to 2.30 pm on the 11/9/99. An analysis of variance demonstrated no significant difference (P = 0.29) between the mean spore germination at 0, 2, or 26 hours. However, there was a suggestion of declining viability with increased exposure to environmental conditions.

Table 6.7: Counts of germinated spores of *B. allii* (out of 200 spores examined) incubated on water agar after exposure to sunlight for different periods, no significant difference found with ANOVA (P = 0.29) LSD = 46.8.

Replicate Petri plates	*Duration of exposure to sunlight*		
	0	*2 hours*	*26 hours*
Plate 1	106	101	51
Plate 2	97	97	73
Plate 3	110	68	96
Average germination (%)	104.3	88.7	73.3

6.4 Discussion

The experiments conducted in this chapter provided information which assisted the understanding and interpretation of results obtained in field trials (Chapters 7 to 9) on the development of epidemics of neck rot in space and time.

6.4.1 Spore trapping

Kritzman's agar in Petri plates was demonstrated to be of potential use as a spore trap. However, it was only suitable for very short trapping periods, as the agar tended to dry quickly when used outdoors (results not shown).

6.4.2 Spore release

By graphing the weather parameters against counts of spores of *B. allii* trapped using a volumetric spore trap, rainfall appeared to be an important factor in *B. allii* spore release in the first two experiments. Rainfall may mechanically dislodge spores from conidiophores. Experiments 1 and 2 demonstrated sharp increases in numbers of spores trapped with the first major precipitation event, and then smaller peaks in spores trapped subsequently. This was perhaps due to the original flush of spores being produced in the laboratory under ideal conditions, with no mechanical force applied during this period so they remained intact on the conidiophores until mechanically dislodged with a precipitation event. The longer smoother peaks noted in Experiment 3 were probably due to a number of factors, such as mechanical dislodgment of spores by wind and rain, and possibly release of spores upon drying of the conidiophores.

The analysis of weather data versus spore release demonstrated that a large amount of environmental and spore data are required to assess the relationships of spore release with weather parameters. Unfortunately, the difficulty in collecting spore release data prevented development of such a model during this project. An improved method of assessing spore trap data is required to collect enough information to develop or modify existing spore release models. Perhaps an immunological method or PCR detection method would assist in collecting such spore release data.

Spores of *B. allii* were collected in rainwater close to a source of *B. allii* conidiophores, suggesting that spores are spread by splashing rain droplets. However, it was also shown that *B. allii* can be released dry into the air as demonstrated by the detection of spores on trap plants and filter traps, as well as the Quest volumetric spore trap, in the absence of rainfall events. The filter traps proved useful as an inexpensive qualitative trap. Unfortunately, they provided no quantification of spore release. Quantifying the likelihood of long-range spread of *B. allii* or any airborne particle is extremely difficult. Improved methods of spore trapping may assist in answering this question. Fungi that produce spores which are readily distinguished microscopically are more amenable to spore-trapping experiments, unlike *Botrytis* spp. which produce nondescript, hyaline, single-celled spores. Immunological or nucleic acid-based methods of detecting the presence of fungi in trapped air, or in plants, may facilitate such experiments with *Botrytis* spp.

Spores dispersed primarily in dry weather can be divided into two categories, those that are actively released or shed under gravity, and those that are removed by

mechanical dislodgment during periods of fast turbulent wind (Aylor, 1990). The *Botrytis* genus belongs to the second category of passive liberation. Conidia of *Botrytis* are often removed as clusters of two or more spores, which increases the settling speed, enhancing deposition close to a source (Aylor, 1990). For these fungi, the direct action of wind, or leaf-flutter and stem-vibration, produced by wind or heavy rain are chiefly responsible for spore liberation (Ingold, 1978). Jarvis (1962) found that spores of *B. cinerea* in a raspberry plantation were prepared for release by hygroscopic movements of the conidiophore, which detached conidia from the conidiophore, producing a spore powder ripe for liberation. Wind movement during the windy mid-day hours liberated the conidia. Jarvis (1962) also noted that during still nights, a heavy rainfall event would also result in high levels of *B. cinerea* conidia being liberated. Heavy rain not only has direct impact effects upon bunches of conidiophores but resulted in leaf vibration, aiding spore release.

Small (1970) stated that fluctuations in RH appeared to be involved in the liberation of conidia of *B. squamosa,* by causing hygroscopic movements of conidiophores. Swanton (1977) added that airborne dispersal of spores of *B. squamosa* showed a marked circadian periodicity. No obvious circadian rhythm was observed in the spore release patterns found in the experiments with *B. allii* conducted in this thesis. Swanton (1977) found peak numbers of *B. squamosa* spores to occur between 0600 and 1200 hours, in association with decreasing relative humidity, increasing temperature and increasing wind speed. Maximum spore numbers were associated with rapid drying of the leaf surface. Similar release patterns were noted for *B. fabae* conidia by Fitt *et al.* (1989). Greatest numbers of *B. fabae* conidia were collected

during periods of heavy rain, and on dry days the greatest number of spores were collected in the morning where wind speed was increasing and humidity was decreasing. The release patterns of *B. allii* noted from the limited data that was collected, would tend to agree with those found for *B. squamosa* and *B. fabae,* mentioned above.

The fact that none of the measured weather parameters completely corresponded with the rise and fall in numbers of *B. allii* spores trapped in the air in this study may be due to several factors:

1. Spore release may result from a combination of all or some of the parameters measured i.e., temperature, relative humidity, rainfall, leaf wetness, wind speed.
2. Spore release may be more clearly related to a factor not measured, e.g., wind direction.
3. The trial period may have been of insufficient duration to gather enough data to identify the complex interaction of environmental factors involved in spore production and release by *B. allii.*

The spore release characteristics from cut bulbs may be different from spore release from plants in a field. A number of factors may influence spore production and release within a crop situation, e.g., the presence of senescing leaves on which spores are readily produced. The cull pile in this experiment would initially have had a large number of *B. allii* spores on it, but the number of spores produced subsequently may have declined as saprophytes began to colonise the cull pile and out-compete *B. allii.* Movement of onion leaves in the wind may also explain why spores are released in

the absence of precipitation events in field trials, whereas precipitation events are needed in bulb cull piles for physical dislodgment of spores.

6.4.3 Spore dispersal gradients and disease gradients

The spore dispersal gradient observed for *B. allii* from the cull pile was very steep. This was supported by the disease gradients of *B. allii* spores observed in the field, suggesting that precipitation events were important in dispersal of this fungus. The inverse power law and the negative exponential law are empirical models that have been used to describe spore deposition gradients for sources of inoculum located near ground-level. The main difference between these two models is that the exponential model implies a constant length scale, where the power law model implies a length scale that increases proportionally with distance. The increase in length scale reflects the main characteristic of turbulent dispersal, i.e., increasingly larger eddies which diffuse the spore cloud (Aylor, 1990). Dispersal by rain splash is generally well described by the exponential model (Fitt *et al.*, 1989), which should also describe dry-spore dispersal in situations where the turbulence is mild and spores are transported mainly within the canopy by air movement (Aylor, 1990). The power law model normally fits situations where dispersal occurs in highly turbulent wind, i.e., where spores are likely to be removed from the canopy. This may be most likely to occur with those types of spores that that are removed during wind gusts. From the regression analysis for *B. allii,* it appears as though the power law model describes spore dispersal from a cull pile slightly better than the exponential model. Although the differences were slight for the two models, further work is needed to determine which model best fits the dispersion of *B. allii* spores under rainfall events. The power

law model also describes the disease gradients (Chapters 7 and 8) within the crop better than the exponential model. This may be due to the canopy structure of the onion crop. If the crop canopy were taller, the same pathogen may have demonstrated a better fit to the exponential model, as spores would be less likely to leave the canopy and more likely to be deposited on neighbouring plants.

There are numerous reports of *B. cinerea* being transmitted by thrips (Fermaud *et al.* 1994), *Drosophila* spp. (Louis *et al.* 1996) and moths (Bailey *et al.* 1997). It is, therefore, possible that onion thrips (*Thrips tabaci*) or other insects within the onion crop may disseminate *B. allii* conidia. Onion thrips is a common pest in Tasmania. However, no information is available on insect transmission of *B. allii.*

6.4.4 Spore deposition

Botrytis allii spores are hydrophobic which, according to Ingold (1978), gives spores an advantage when they are carried by rain splash droplets. Spores that are hydrophilic can be taken into the raindrop, but those that are not wettable will cling to the outside of the droplet surface. When a drop runs off a leaf surface, spores that are on the outside of the droplet will be deposited on the leaf surface. However, those spores that have been taken into the raindrop will run off with the rain drop (Ingold, 1978). This means that *B. allii* may be spread well by rainsplash, resulting in short range spread, which is reflected in the disease gradients noted in the Field Trials (Chapters 7 and 8).

6.4.5 Spore germination and plant infection periods

The spore germination experiments demonstrated that the spores of *B. allii* require between 3 and 16 hours to germinate. Free water is required for this process, as spores did not appear capable of germinating at 100% relative humidity. Trap plants in the field demonstrated that plant infection conditions were present in all 24-hour periods tested. The number of trap plants infected with *B. allii* correlated significantly with the number of filters that tested positive for *B. allii.*

Germination of spores *in situ* would be a good way to study spore germination and determine the required environmental factors for plant infection. Plant growth chamber studies would be ideally suited for this. However, growth chambers were not available during the course of this study.

6.4.6 Spore survival as affected by sunlight exposure

Rotem and Aust (1991) stated that the UV spectrum of sunlight is the most important environmental factor influencing mortality of fungi. Walter *et al.* (1999) demonstrated that the conidia of *B. cinerea* remained viable on the surface of kiwi fruit throughout the growing season in New Zealand. This may have been due to the effect of shading from the canopy and the hairy nature of the kiwi fruit, as Rotem and Aust (1991) demonstrated that *B. cinerea* was sensitive to sunlight in a study in Israel. Rotem and Aust (1991) found that single spores of *B. cinerea* did not survive more than 2 days of exposure to sunlight, and no longer than 5 days when the spores were in clumps.

The pilot trial in this thesis demonstrated that exposure to sunlight did begin to decrease the germination potential of *B. allii* spores, although this was not statistically significant between periods of 0, 2 and 16 h. Further examination of this is required to determine the limitation of exposure to sunlight on long-distance spread of the pathogen via wind currents. Experiments will need to be run over different times of the year to check for seasonal affects (i.e., increased UV levels, or temperatures during summer vs. winter). However, the results from this study demonstrated that spores of *B. allii* are capable of surviving exposure to sunlight. The length of survival of *B. allii* conidia will most likely depend upon where the conidia are located. If in the shade, survival of spores will undoubtedly be longer than for those exposed to direct sunlight. The Petri plate plastic covers may have reduced the intensity of ultra-violet light reaching the conidia and reduced desiccation. Further experiments are needed to determine the importance of sunlight on *B. allii* spore survival.

6.4.7 Future work

Immunological techniques have been utilised in the detection of certain fungi and other aerial micro-biota. Such techniques include the direct visual identification of spores through immuno-fluorescence microscopy (Tijs Giles, *personal communication*), image analysis techniques, or ELISA systems to quantify spores in air samples (Kennedy *et al.,* 2000). PCR techniques can also be used for the identification and enumeration of fungal or aerial micro-biota (Hernandez *et al.*, 1999). Antibody-based or PCR methods for routine detection of small numbers of *B. allii* spores may assist in understanding the windborne spread of this fungus. Unfortunately, at present there are no monoclonal antibodies for use in identifying *B.*

allii spores. Polyclonal antisera have been developed for the detection of *B. allii.* However, polyclonal antisera may cross-react with other *Botrytis* isolates (Linfield *et al.,* 1995; Mackie, 1997). At present, we have to rely on visual identification of spores or the use of culture techniques. Recently, *B. allii*-specific PCR primers have been developed (Nielsen *et al.*, 2002) which may offer a means of specific detection of *B. allii* spores. However, a protocol for use on spore trap samples needs to be developed before this method can be used routinely for such purposes.

Spore monitoring and fungicide application forecast systems have been developed relatively successfully for *B. squamosa* (Shoemaker and Lorbeer, 1977a and 1977b; Sutton *et al.*, 1986; Alderman *et al.*, 1987; Vincelli and Lorbeer, 1989; de Visser, 1996). *Botrytis squamosa* is a foliar pathogen that typically causes symptomatic infection on onion leaves and is favoured by well defined weather events (warm humid weather) thus making it easier to monitor than *B. allii.*

In the future, monitoring techniques may allow strategic control strategies to be implemented for *B. allii.* One possible detection system that is currently being developed for fungal pathogen systems is the microtitre immunospore-trapping device (MTIST) (Kennedy *et al.*, 2000). The MTIST traps fungal spores by impaction of spores in microtitre wells. The ELISA technique can then be used to quantify target spores given an appropriate antibody is available. An MTIST system could be used to apply fungicides strategically when *B. allii* spores are released and environmental conditions are conducive for infection. Other detection systems such as PCR assays

may also be of use in the future, although at present the cost of such systems is often prohibitive (Nielsen and Yohalem, 1999)

Chapter 7

7 Modelling the spatio-temporal spread of *Botrytis allii* in onion crops (Field trial 1)

7.1 Introduction

It is difficult to assess infection of onions by *B. allii* in the field because of the absence of obvious symptoms. Because of this, little information is available on the mode, rate and distance of spread of *B. allii*. Gathering information on a *B. allii* epiphytotic is usually reliant upon assessing the final disease incidence that occurs in stored onion bulbs. This provides little information on the dynamics of the epiphytotic that occurs throughout the season.

The aim of this trial was to characterise the temporal and spatial patterns of spread of *B. allii*, and to monitor disease progress over the course of an epiphytotic. This is probably the first trial examining an epiphytotic of *B. allii* throughout the season. Through observing patterns of spread in time and space, insight into dispersal of the fungus and conditions necessary for the epiphytotic to develop can be gained. Knowledge of the characteristics of *B. allii* epiphytotics will be useful in assessing and developing control strategies and in the planning of future research.

7.1.1 Temporal analysis

The plot of disease incidence with time, the disease progress curve[1], has been referred to as the "signature" of the epiphytotic. The disease progress curve represents the interactions of all factors involved in an epiphytotic, i.e., the pathogen, host and environment (Campbell and Madden, 1990). Characteristics of a disease epiphytotic can be investigated through analysis of disease progress curves, including time of disease onset; rate of disease increase; shape of the disease curve; initial, maximum and final amount of disease and duration of the epiphytotic (Bowers and Kinkel, 1997). The disease progress curves most commonly fitted are the monomolecular, exponential, logistic, Gompertz and linear population growth models as explained in the Literature review (Chapter 1).

7.1.2 Spatial analysis

7.1.2.1 Epiphytotic mapping

Mapping is one of the most widely applied techniques for spatial pattern analysis (Campbell and Madden 1990). It is simple to perform and provides a rapid visualisation of patterns that may be present. Maps may be constructed in either 2 or 3 dimensions. Some form of mapping should be used as a first step in any spatial pattern analysis (Campbell and Madden 1990). Because infection of plants by *B. allii* seldom produces symptoms in the field, constructing a map of asymptomatic infection gives insight into the otherwise invisible spatial pattern of infection.

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[1] Although *B. allii* rarely produces symptoms of disease in the field, the conventional terminology 'disease progress curve' is used in this thesis to signify the increase in incidence of infected plants with time.

7.1.2.2 Spore deposition gradients and disease gradients

An introduction to spore deposition and the analysis of disease gradients was given in chapter 1 Section 1.7.3.

7.1.2.3 Tests for spatial pattern

A number of spatial analysis methods were used to analyse the distribution of infected plants in the field trial in this study including ordinary runs analysis, beta-binomial distribution, spatial analysis by distance indices (SADIE), and radial correlation analysis. A variety of statistical tools were used in this study to provide comparisons between the methods. A brief summary of each of the methods follows. A more detailed account was presented in the literature review.

Ordinary runs analysis determines if the arrangement of a succession of binary events, defined here as the infection status of plants or quadrats, are distributed randomly or non-randomly, usually in one-dimension (i.e., within the rows) (Campbell and Madden, 1990).

Fitting the data to both the beta binomial and binomial distributions was done using the BBD program (Madden and Hughes, 1994). A good fit to the binomial distribution is suggestive of a random spatial pattern. A good fit to the beta binomial distribution suggests an aggregated spatial pattern of disease incidence (Turechek and Madden, 1999).

Spatial analysis by distance indices (SADIE) determines if an observed spatial pattern is random, by measuring the minimum total effort required to move each of the

individuals in the observed sample so that all are arranged as uniformly or regularly as possible (Perry and Hewitt, 1991). The effort for the observed individuals to move to regularity is quantified as the distance to regularity (Perry, 1995b). The degree of non-randomness can then be quantified by comparing the observed spatial pattern with rearrangements in which the sample counts are randomly permutated amongst the sampled locations.

The radial correlation analysis used in this study was performed by Dr Frank Ferrandino (Connecticut Agricultural Experiment Station, New Haven, Connecticut, USA), using a derivative of the program 2DCORR, created by Dr Ferrandino (Ferrandino, 1998). The program accounts for differences between reference and target plant populations and calculates the fraction of the actual to expected number of infected plant pairs in every direction and distance. Radial correlation analysis judges correlations over and above trends already present in the field, such as edge effects or anomalous distributions.

7.2 Materials and methods

7.2.1 Field trial preparation

A soil sample of the trial area was taken on the 8/6/99 and analysed at Mt Pleasant Laboratories, Department of Primary Industry, Water and Environment (

Table 7.1). The agricultural consulting company Serve-Ag PTY. LTD. also completed a soil nitrate test prior to planting and found a level of 84.5kg N/ha.

Table 7.1: Field trial 1 soil test, conducted at Mt Pleasant Laboratories, Department of Primary Industry, Water and Environment.

Property tested and method	Result
pH (1:5 soil:water)	5.8
Total N	2.8%
Phosphorous (Colwell)	80.6 ppm
Potassium (NH_4OAc extract)	226 ppm

The field trial was established on the 25/6/99, with seed of the variety 'Creamgold' (line 9-R-210) certified free from infection by *B. allii* by the Department of Primary Industries Water and Environment (DPIWE) diagnostic laboratories, New Town, Hobart. The seed test for certification undertaken by DPIWE was a standard seed test of 550 seeds and failed to detect infection with *B. allii.* The test was the method described in Appendix 12.4. However, half strength lactic acid potato dextrose agar (Appendix 12.5.3) was used instead of pectin agar. A subsequent test of 550 seeds conducted by the author on pectin agar also failed to detect *Botrytis* spp.

The trial area was 25 m x 75 m and consisted of 15 beds of onions with 9 rows per bed. The trial was orientated with the 75 m length of beds running in an east-west direction. The width of each bed was 1 m, and beds were separated (between beds) by 0.6 m alleys. The seed was supplied and drilled by Field Fresh Tasmania Pty. Ltd. The seed did not have any fungicidal seed treatments and was sown at a density of 5.5 kg/ha, with an Accord precision drill. Plant density was measured at the 1st true-leaf stage. On average, there was 0.07 m between plants down the rows, with a distance of 0.125 m between rows within beds. The trial was part of a larger crop of onions. The paddock had never been planted to onions before and the previous crop was pasture

(ryegrass/clover). The trial was fertilised and managed with pesticides as for a commercial crop (Table 7.2 and Table 7.3).

Table 7.2: Fertilisers applied to Field Trial 1

Date of application	Fertiliser
Prior to sowing	2.5 t/ha lime + 2.5 t/ha dolomite 600 kg/ha 9.13.17 +0.1% Zn, 0.1% Cu, 0.2% B, 0.02% Mo 100 kg/ha muriate of potash 700 kg/ha double super phosphate
25/6/99	125 kg/ha triple super phosphate drilled with seed
26/10/99	Urea 90 kg/ha
16/11/99	Urea 100 kg/ha
20/12/00	Urea 125 kg/ha Potash 125 kg/ha

Table 7.3: Pesticides applied to Field Trial 1

Date of application	Pesticide
17/7/99	Roundup[1] 2 L/ha Stomp[2] 1 L/ha Lorsban[3] 0.7 L/ha Activator 120 ml/ha
24/8/99	Stomp 2 L/ha
9/9/99	Verdict[4] 200 ml/ha
3/10/99	Tramat[5] 600 ml/ha
16/10/99	Tramat 800 ml/ha
19/10/99	Totril[6] 600 ml/ha Tribunil[7] 600 g/ha
25/10/99	Tortril 750 ml/ha Bladex[8] 750 g/ha

[1]Glyphosate, [2]Pendimethalin, [3]Chlorpyrifos, [4]Haloxyfop-methyl (unstated stereochemistry), [5]Ethofumesate, [6]Ioxynil, [7]Methabenzthiazuron, [8]Cyanazine.

The trial was irrigated with water from a rain-fed dam, using a solid-set irrigation system. The output of the sprinklers was 21 mm per hour. Sprinklers were placed between beds 2 and 3, 7 and 8, and 12 and 13; with 5 sprinklers placed between beds 2 and 3, and 12 and 13, and 6 sprinklers placed between beds 7 and 8. There was a

distance of 10 m between sprinklers within the beds. Sprinklers irrigated a radius of 7 m and were staggered to provide maximum coverage of the crop.

7.2.2 Weather data collection

Weather data was collected using a Unidata Starlog data logger (manufactured by Unidata Australia, 3 Whyalla St, Willetton 6155, WA) with external probes for rain, temperature, percentage RH and leaf wetness measured on a 0-2550 mV scale (dry to wet, 0 being dry and 2250 being completely saturated). The data logger was placed in the middle of the trial area, within a bed. These parameters were recorded every hour.

7.2.3 Trial inoculation

Onion bulbs from commercial crops infected with *B. allii* were used as inoculum. The trial was exposed to inoculum on the 22/11/99, by placing six infected bulbs in a 1 m^2 area on the outside corner of the trial. The inoculum source was replenished on the 26/11/99 with six more infected bulbs.

7.2.4 Sampling procedure

On the 9/11/1999 a sample of 225 leaves was collected randomly from across the trial site to determine if there was any *B. allii* infection prior to placement of inoculum. To provide information on the spatial pattern of *B. allii*-infected plants in subsequent sample periods, the trial was partitioned into 100 quadrats arranged in a 10 x 10 lattice. Each quadrat was 1 bed wide (1.6 m) by 4.48 m long. Within each quadrat, 33 plants arranged in a fractal pattern were tagged at the 3-leaf stage with flagging tape which was placed loosely around the base of the plant for the entire sampling period. The sampling pattern is illustrated in Figure 7.1, showing four quadrats of plants. A

fractal sampling pattern was chosen because of the advantage it produces in ensuring the collection of samples at various plant-pair distances, which are used in the analysis of spatial pattern by radial correlation (F. Ferrandino, *personal communication*). Within each quadrat, plants within the row were tagged at 0.07, 0.14, 0.21, 0.35, 0.49, 0.77, 1.05, 1.61, 2.17, 3.29 and 4.48 m from the origin in each of rows 1, 2 and 7 within each bed of 9 rows. This translated to plant numbers 1, 2, 3, 5, 7, 11, 15, 23, 31, 47 and 64 within each row sampled. Therefore at each of the 5 sample times, 33 leaves (1 per plant) were removed from each plot (i.e., 3300 from the trial).

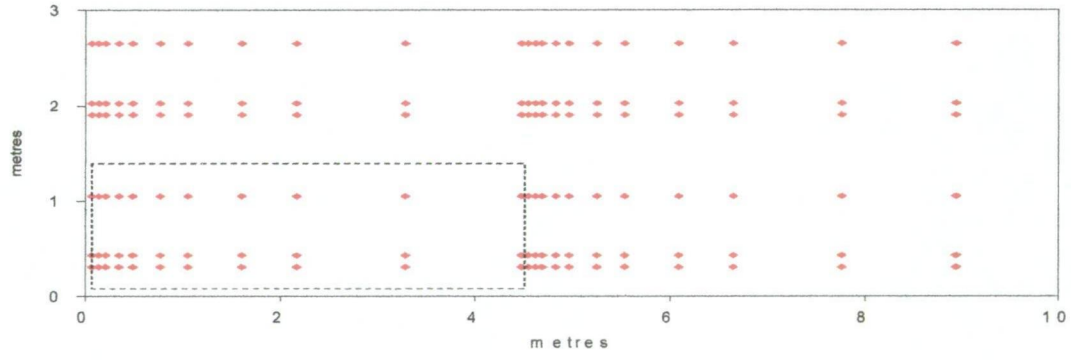

Figure 7.1: Fractal sampling pattern used in Field trial 1, with four quadrats shown (broken line indicates one quadrat)

At each sample time, the sampling unit consisted of the oldest green leaf. Dry leaves were not collected. Each leaf was placed into an individual marked paper bag for transport back to the laboratory within 3 hours of collection. The sampling was performed five times over the growth of the crop (Table 7.4).

Table 7.4 : Sampling schedule for Field trial 1

Date	*Crop age (days)*	*Event*
25/6/99	0	Crop sown
9/11/99	137	Random leaf sample taken (225 leaves)
22/11/99	150	Inoculum placed
13/12/99	171	First spatial leaf sample
4/1/00	193	Second spatial leaf sample
20/1/00	209	Third spatial leaf sample
4/2/00	224	Fourth spatial leaf sample
14/2/00	234	Fifth spatial leaf + bulb sample
15/2/00	235	Onions bulbs lifted
6/3/00	255	Onion bulbs put into storage
31/5/00	341	Onion bulbs assessed (106 days from lifting)

7.2.5 Sample assessment

Leaf samples were placed into individual marked 1 L-food containers with a wet tissue in the bottom. A lid was placed onto the container to maintain high humidity and the containers were stacked on the bench in the laboratory for 7 days (Appendix 12.8). The leaf samples were assessed for *Botrytis* spp. with the aid of a strong light. A dissecting microscope (50X) was used to differentiate between conidiophores of *B. allii* and *B. cinerea.* Where necessary, species differentiation was confirmed by isolating onto pectin agar in petri dishes (Appendix 12.7). This occurred where there was limited growth of *Botrytis* conidiophores, or where it was difficult to differentiate conidiophores as those of *B. allii* or *B. cinerea.* The incidence of both *Botrytis* species (*B. allii* and *B. cinerea*) was recorded.

To assess disease development, 33 bulbs were collected from each of the 100 quadrats on the 14/2/00. Bulbs were collected immediately adjacent to the tagged plants from

which leaf samples had been gathered within rows. Bulbs were stored for 15 weeks from the lifting date in an insulated shed in 0.5 t wooden crates. Ambient temperature in the shed ranged from 10 to 15°C and the RH ranged from 65 to 98%. After storage, the onions were visually assessed for symptoms of *Botrytis* neck rot (as defined in Chapter 3) by cutting the bulbs in half lengthwise.

7.2.6 Data analysis

To visualise and analyse the spatial and temporal spread of the pathogen through the crop, disease maps, disease progress curves and disease gradients were produced. Spatial patterns were assessed using ordinary runs analysis, beta-binomial distribution analysis, spatial analysis by distance indices (SADIE) and radial correlation analysis. Methods of temporal and spatial analysis are discussed in the sections that follow.

7.2.7 Disease maps

Disease maps were prepared by entering XY position coordinates for each infected plant. Microsoft Excel® was used to construct the maps. Successive sampling data points were overlaid on the previous map, so that once a plant was infected, it was assumed to remain infected for the duration of the trial. Cumulative disease maps were prepared for both *B. allii* and *B. cinerea*. The graphical software, Sigmaplot® was also used to interpolate between points and plot the contours of the final cumulative quadrat data sets for both species. Two methods were used to develop contour plots of *B. allii* infection incidence using Sigmaplot®. The first method used the values of percentage infection on a quadrat basis, resulting in a smooth contour plot of areas of infection. The second method used the individual sample plant information, which contoured each individual focus.

7.2.8 Foci expansion – infection front

To examine the edge of the expanding infection front, the distance from the inoculum source to the furthest *B. allii*-infected plant was determined and plotted against time. The plot was then analysed by regression analysis using the statistical software Genstat® Version 4.1.

7.2.9 Disease progress curves

Disease progress curves were prepared by plotting the number of plants infected as a percentage of the total number of plants present in the entire sample per sampling period, i.e., 3300 plants. The cumulative and non-cumulative incidence of *B. allii* and *B. cinerea* was analysed for goodness of fit to the following population growth models: linear, exponential, monomolecular, logistic and Gompertz (discussed in literature review, Section 1.7.2) (Table 7.5), using a Statistical Analysis Software® (SAS®) macro version 8.0 (Campbell and Madden, 1990). The cumulative data was based on the assumption that once a plant was identified as infected, it remained so for the duration of the trial, while the non-cumulative data was based on the actual incidence of infected plants detected at each time of sampling. The SAS package provided graphical outputs of the observed and expected disease incidence values for each of the models, and the predicted and residual errors in disease incidence. Each model was evaluated for its fit to the actual data using the graphical outputs, F-probability, R^2 and mean square error (MSE) values. To compare R^2 values directly between models, a back transformation was performed.

Table 7.5: Growth models and their formulae (Campbell and Madden, 1990)

Model	Integrated equation
Monomolecular	$y = 1-(1 - y_O)\exp(-r*t)$
Exponential	$y = (y_O)\exp(r*t)$
Logistic	$y = 1 / \{1 + [(1 - y_O)/y_O]\exp(-r*t)\}$
Gompertz	$y = \exp(\{[\ln(y_O)]\exp(-r*t)\}$
Linear	$y = y_O + r*t$

y = incidence of infected plants at time of observation

y_O = incidence of infected plants at first observation

r = rate of disease increase for a specific model

t = epidemic duration

7.2.10 Disease gradients

Disease gradients were determined by plotting the incidence of infected plants at a range of distances from the inoculum source, using two methods. For the first method, the percentage incidence of infection in each quadrat was assumed to be located at the centre of each quadrat. The distance from the centre of the quadrats to the plot corner (point source) was graphed against the incidence per quadrat (Gregory, 1968). For the second method, the incidence of infected plants within metre annuli from the point source was mapped (Gregory, 1968). A count of the number of sampled plants in each annulus was made in addition to the number of infected plants, so that the percentage infected plants could be determined within each metre annulus. Infection gradients from the inoculum source across and within beds were also plotted. Distance was determined from the corner of the plot to the centre of the quadrats or annuli. Microsoft Excel® was used to graph the gradient.

Analysis of the infection gradients was undertaken using the software Genstat® Version 4.1. The gradients were fitted to exponential and power law models (Table 7.6). The Genstat code input file is included in Appendix 12.12.

Table 7.6: Empirical models used in the analysis of disease gradients

Model	Formulae
Exponential model	Proportion +ve = A x exp (-B x distance)
Power law model	Proportion +ve = A x distance^{B}

A (y-axis intercept) and B (slope) are parameters. Proportion +ve = the proportion of infected plants at a set distance.

7.2.11 Spatial analysis of *B. allii* and *B. cinerea* leaf infection and *Botrytis* bulb rot

Spatial analysis techniques and method were discussed in detail in the literature review in Section 1.8. Spatial analysis of data sets with disease incidence greater than 95% or less than 5% was not undertaken as the standard-normal approximation for the test statistic under the null hypothesis of randomness may (or may not) hold in these circumstances (Sarah Pethybridge, Tasmanian Institute of Agricultural Research, Burnie, *personal communication*). As the incidence of *B. allii* over the entire trial area was not above the 5% threshold for performing spatial analysis, the data set was modified by removing quadrats in the half of the trial furthest from the inoculum source. This resulted in an area of interest comprising 5 quadrats long in the north-south direction, and 10 quadrats wide in the east-west direction. The incidence of *B. allii* in this area was 7% at 84 days after inoculation.

7.2.12 Ordinary runs analysis (ORA)

Ordinary runs analysis (ORA) was used to analyse the proportion of quadrats within and between rows exhibiting significant (P = 0.05) aggregation of infected plants at the 5th sample time (84 days after application of inoculum). If a quadrat contained infected plants it was assigned a 1. If there were no infected plants, the quadrat was assigned a 0.

7.2.13 Beta Binomial Distribution (BBD)

The BBD software (Madden and Hughes, 1994) was used to fit the binomial and beta binomial distribution (BBD) to the spatial patterns of *B. allii* and *B. cinerea* infected plants. For this analysis, the data was arranged as *B. allii* or *B. cinerea* infected plant counts per quadrat. As the incidence of *B. cinerea* infection was above 5% in most cases, the full 100 quadrats of the trial were analysed for this species.

An index of dispersion (D) was produced by dividing the observed variance of diseased plants per sampling unit by the theoretical variance for a binomial distribution. A value of D greater than 1 indicated aggregation (Madden and Hughes, 1994).

A log ratio test statistic (LRS) was used to determine if the BBD was a better fit than the binomial distribution. LRS has a χ^2 distribution with one degree of freedom, under the null hypothesis of no difference between the two log-likelihoods (Turechek and Madden, 1999), the equation is demonstrated below:

LRS = 2 x (log-likelihood of beta binomial – log-likelihood of binomial)

7.2.14 Spatial Analysis by Distance IndicEs (SADIE)

The percentage of leaves and bulbs with *B. allii* and leaves with *B. cinerea* were analysed with SADIE using a seed number of 2104 and 153 simulations. The index of aggregation (I_a) and the probability (P_a) that the observed counts were arranged randomly among the sample units were tabulated from the SADIE output files. Clustering indices were also plotted and contoured using Sigma-plot® (Version 7). Confidence intervals of 90 and 95% were used to demonstrate clusters of infected plants and gaps of infected plants determined by SADIE, at P = 0.10 and P = 0.05 respectively.

A binary data set of individual plant infection status was prepared from the cumulative leaf incidence of *B. allii* (i.e., assuming that once a plant had been found to be infected it was considered to remain infected in subsequent sample periods). A maximum of 500 samples could be processed using the SADIE program, therefore only plants close to the inoculum source were used. This included plants from 0 to 5 m along the west axis and 0 to 15 m along the north axis.

The incidence of *B. allii* in leaves and bulbs in the 50 quadrats closest to the source of inoculum was analysed to ensure an incidence above 5%. The incidence of *B. cinerea* in leaves in the 50 quadrat sample area was also analysed.

7.2.14.1 Spatial association between *B. allii* and *B. cinerea* and between *B. allii* leaf incidence and *Botrytis* bulb rot

The cluster indices output files from SADIE were used as input files for association analysis (Perry and Dixon, 2002). Using the spatial correlation program, a value of

association was determined for each corresponding cluster index between the data sets examined. Spatial associations between *B. allii* and *B. cinerea* and between *B. allii* and *Botrytis* bulb rot were tested. The index of spatial association was then plotted for the association at the 95% and 90% confidence levels (P = 0.05 and P = 0.10) for any gaps or clusters in spatial association (Perry and Dixon, 2002). The association program incorporates Dutilleul adjustments to the critical values (confidence levels) and to the overall measure of correlation to allow for any reduction in effective sample size caused by autocorrelation.

7.2.15 Radial correlation analysis

A total cumulative data set of the incidence of *B. allii*-infected plants over the 5 sampling periods was analysed by radial correlation analysis using the software 2DCORR developed by Dr Frank Ferrandino (The Connecticut Agricultural Experiment Station, New Haven, Connecticut, USA). In the plots of normalised density of infected individuals, values between –1 and 1 indicated that the distance class data did not differ from complete spatial randomness (CSR) at the 0.05 α level. Values greater than 1 demonstrated an aggregated departure from CSR and values less than –1 indicated exclusionary departure from CSR each at the 0.05 α level. As previously described, the trial area was reduced by half to increase the total incidence of infected plants to > 5%. Analysis was conducted in conjunction with Dr Ferrandino. To check the distribution of *B. allii*-infected pairs away from the immediate point source, the first two beds of data were removed from the data set before analysis of spatial patterns. A Kolmogorov-Smirnov (KS) statistic was not calculated, as the counts were too low. To test whether aggregation in the field was due to the heavily-infected beds near to the source, the first two beds of high *B. allii*

incidence were removed and the data set was re-analysed. Unfortunately, the expectation values for radial correlation results were less than 5 for many distance classes, so care was required with the interpretation of results. Spatial distribution of *B. cinerea* was not analysed using this technique.

7.3 Results

The sample of 225 leaves gathered at random across the trial prior to placing the inoculum demonstrated that there was no detectable *B. allii* infection prior to the placement of inoculum (Table 7.7). This confirmed the absence of *B. allii* inoculum in seed as indicated by the *in vitro* seed tests. The incidence of *B. allii* in leaves increased from 0.2% at 21 days after placement of the inoculum to 3.8% at 84 days (Table 7.7). The incidence of *B. cinerea* in leaves increased from 0.7% to 38.6% at 21 and 84 days, respectively (Table 7.7).

Table 7.7 : Schedule of events for Field trial 1

Date	Crop age (days) and (days since inoculum placement)	Event	*B. allii* (%)[1]	*B. cinerea* (%)[1]
25/6/99	0	Crop sown	-	-
9/11/99	137	Random sample taken	0	0
22/11/99	150 (0)	Inoculum placed	-	-
13/12/99	171 (21)	First spatial leaf sample	0.15	0.73
4/1/00	193 (43)	Second spatial leaf sample	0.27	4.76
20/1/00	209 (59)	Third spatial leaf sample	1.00	19.30
4/2/00	224 (74)	Fourth spatial leaf sample	2.52	30.81
14/2/00	234 (84)	Fifth spatial leaf + bulb sample	3.79	38.58
15/2/00	235 (85)	Onions lifted	-	-
25/2/00	245 (95)	Bulb sample for coring	-	-
6/3/00	255 (105)	Onion bulbs put into storage	-	-
31/5/00	341 (191)	Bulb storage sample assessed (106 days after lifting)	3.62 attributed to *Botrytis* rot	-

[1]Number of infected plants as a percentage of total # plants sampled.

7.3.1 Disease maps

The spatial positions of the infected plants over the sampling times (Figure 7.2 to Figure 7.6) demonstrate a primary focus of *B. allii* infection developing around the point source of infected bulbs. Infected plants were detected up to 44 m from the point source in the last sampling at 84 days after placement of inoculum (Figure 7.6). The most northern and most western edges of the trial are demonstrated in the maps.

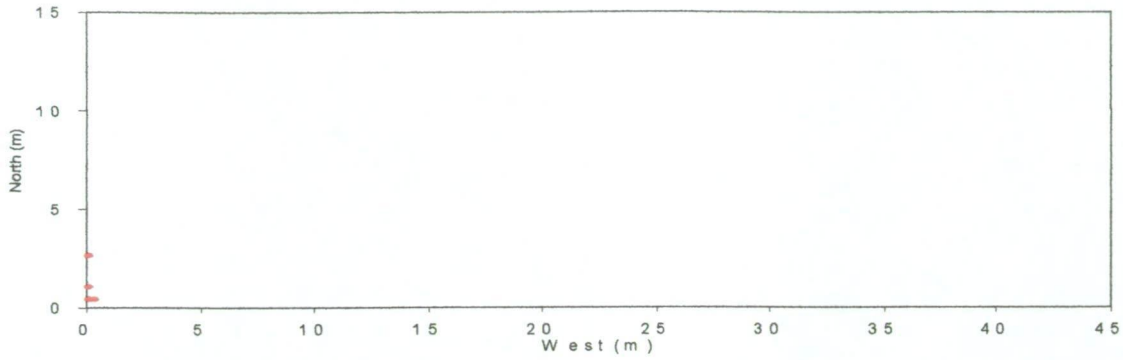

Figure 7.2: Spatial pattern of *B. allii*-infected plants at 21 days after introduction of inoculum (spatial sample 1)

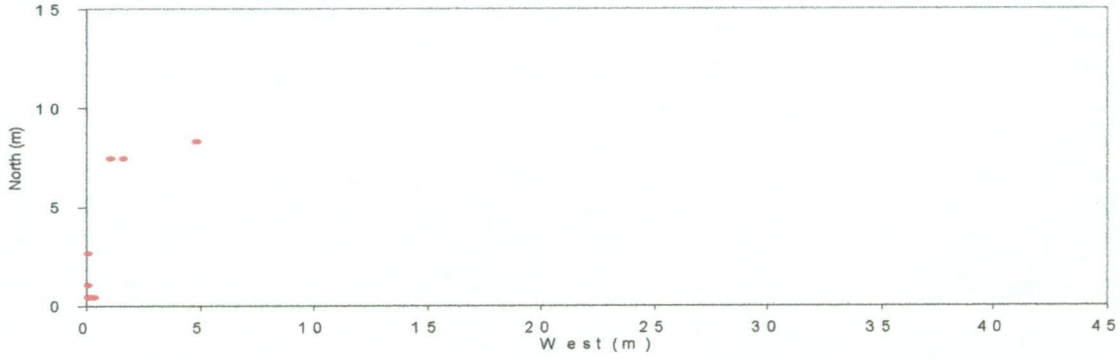

Figure 7.3: Spatial pattern of *B. allii*-infected plants at 43 days after introduction of inoculum (spatial sample 2)

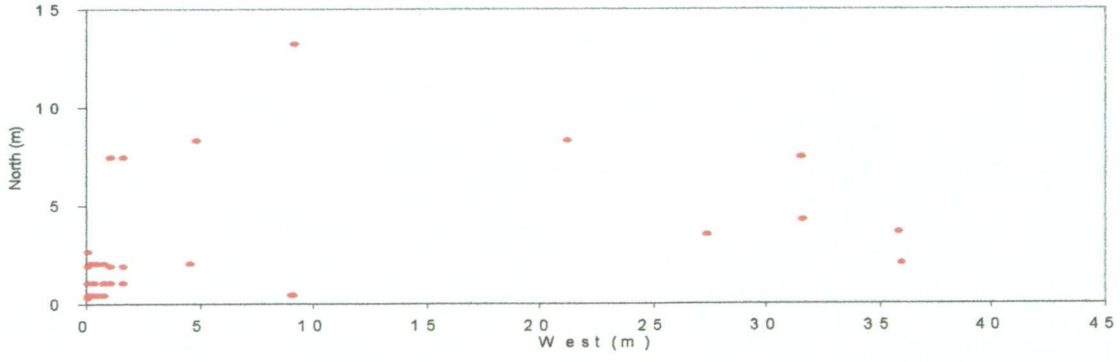

Figure 7.4: Spatial pattern of *B. allii*-infected plants at 59 days after introduction of inoculum (spatial sample 3)

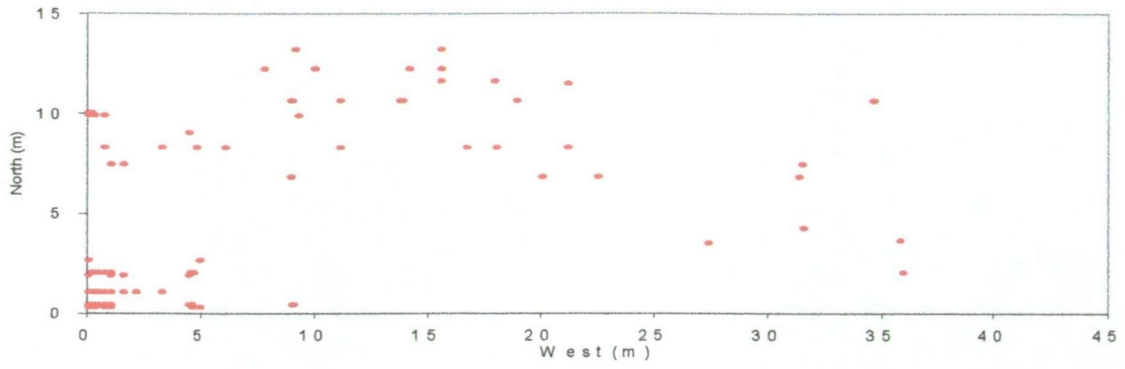

Figure 7.5: Spatial pattern of *B. allii*-infected plants at 74 days after introduction of inoculum (spatial sample 4)

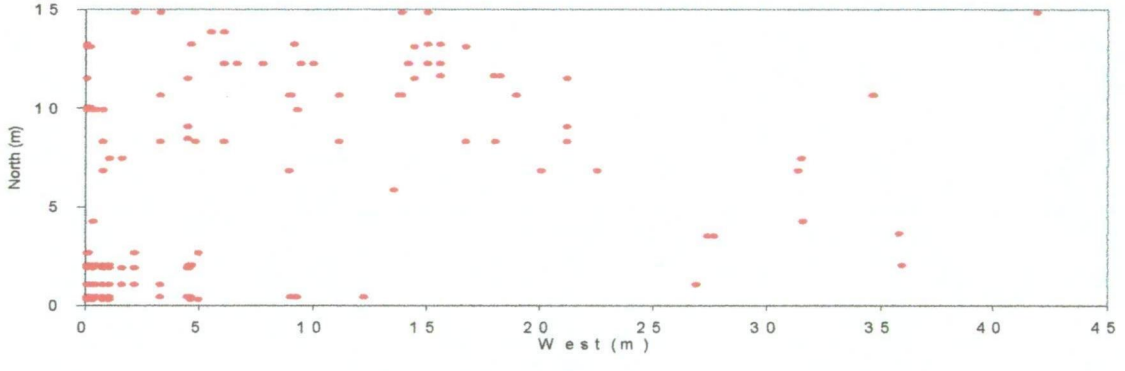

Figure 7.6: Spatial pattern of *B. allii*-infected plants at 84 days after introduction of inoculum (spatial sample 5)

Contour plots of *B. allii*-infected plants are displayed in Figure 7.7 and Figure 7.8. The first plot demonstrates contours based on percentage quadrat infection (Figure 7.7) and the second is a contoured plot of individual infection status expressed as a proportion of infected individuals (Figure 7.8).

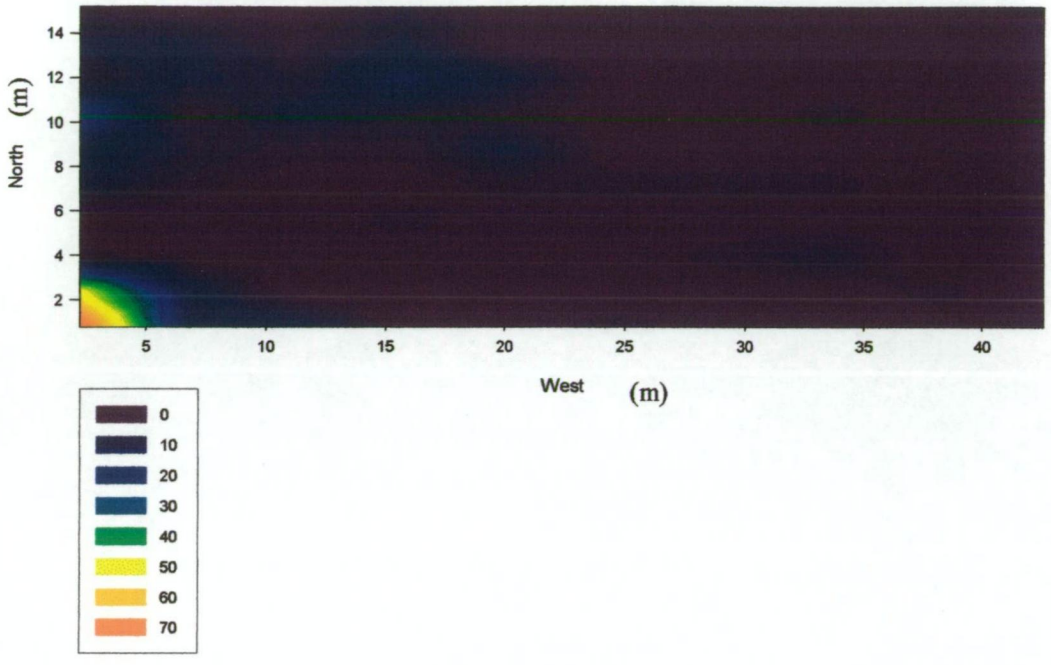

Figure 7.7: *Botrytis allii* incidence contour map based on quadrat percentages (84 days after introduction of inoculum). Where the legend represents the percentage of plants infected: 0, 1-10, 11-20%, etc.

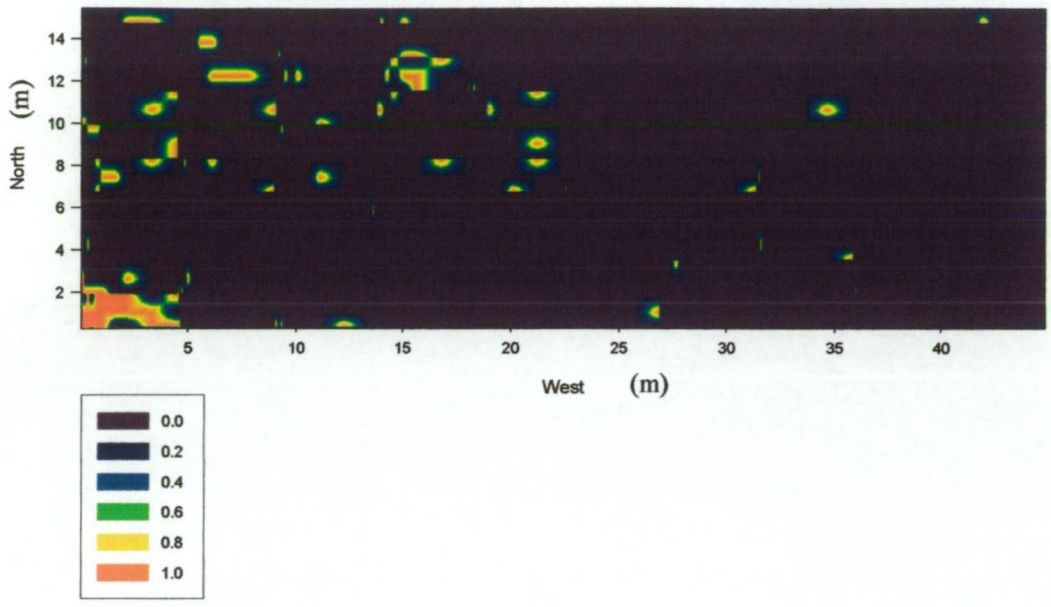

Figure 7.8: Contour map of *B. allii* incidence based on individual plant infection status (84 days after introduction of inoculum). Where the legend represents the proportion of plants infected (proportions are used instead of percentages as map is based on individual plant infection status).

Maps of *B. cinerea* infection qualitatively suggested the pathogen entered the crop randomly (Figure 7.9). Maps of subsequent *B. cinerea* infection demonstrate the rapid increase in the amount of *B. cinerea* infection during the season (Figure 7.9 to Figure 7.13).

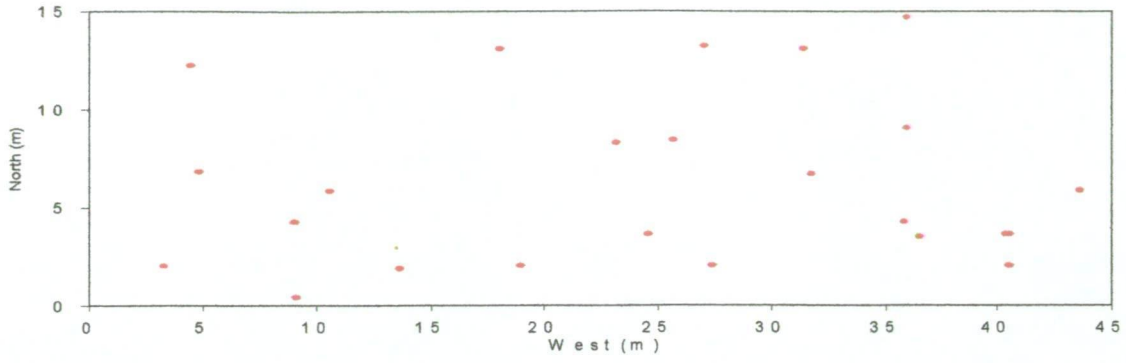

Figure 7.9: Spatial pattern of *B. cinerea*-infected plants at 171 days after planting (spatial sample 1).

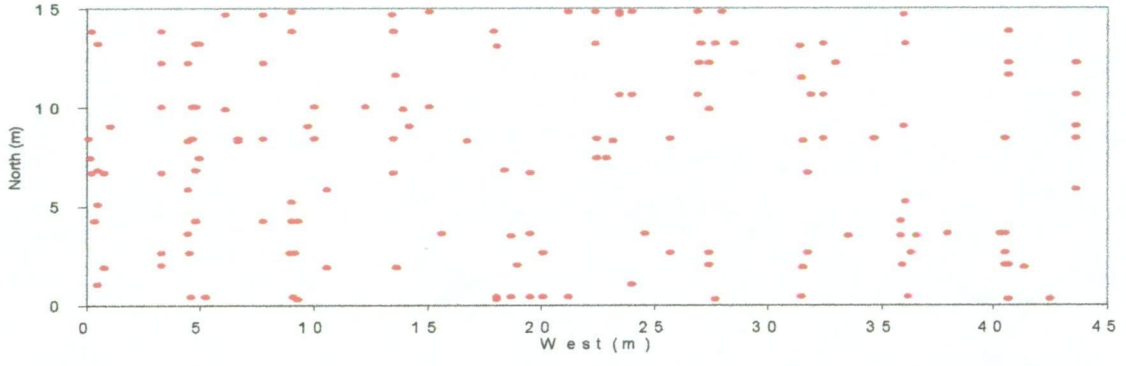

Figure 7.10: Spatial pattern of *B. cinerea*-infected plants at 193 days after planting (spatial sample 2).

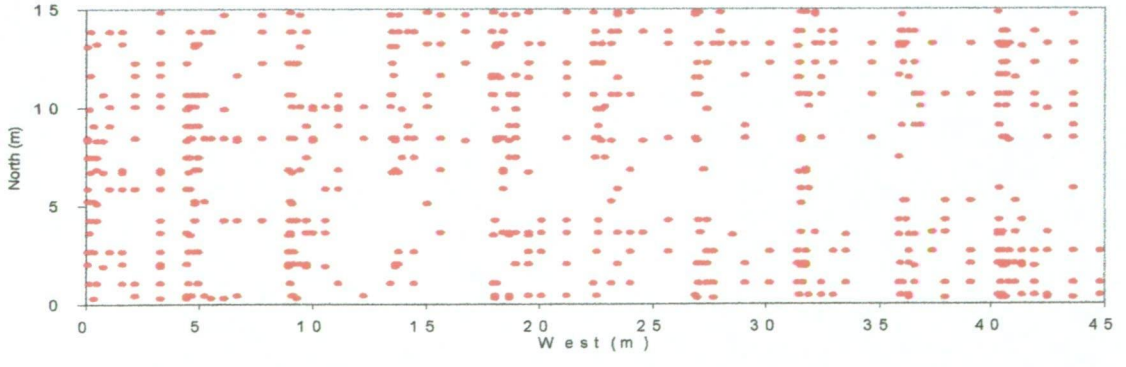

Figure 7.11: Spatial pattern of *B. cinerea*-infected plants at 209 days after planting (spatial sample 3).

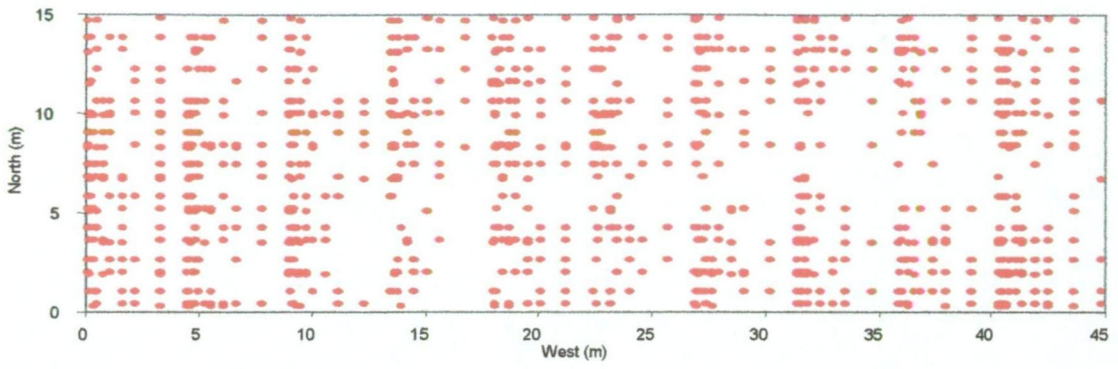

Figure 7.12: Spatial pattern of *B. cinerea*-infected plants at 224 days after planting (spatial sample 4).

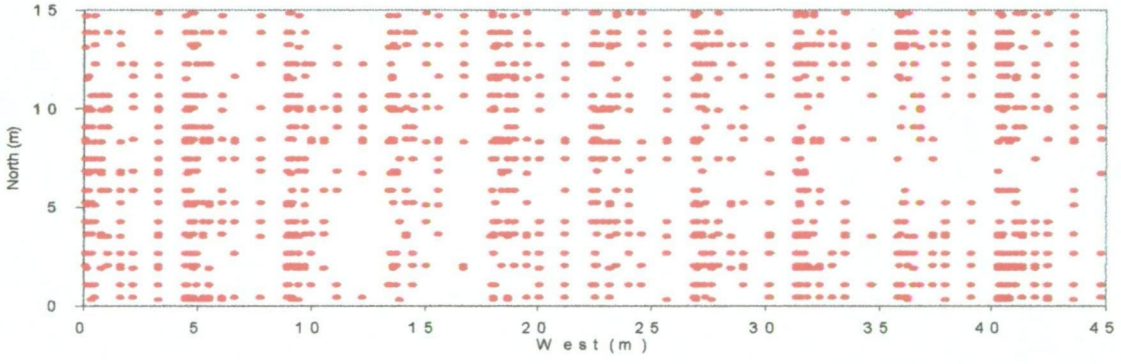

Figure 7.13: Spatial pattern of *B. cinerea*-infected plants at 234 days after planting (spatial sample 5).

Figure 7.14 interpolates the incidence of plants infected by *B. cinerea* at 234 days after sowing, demonstrating areas of high and low incidence that occurred throughout the sample area.

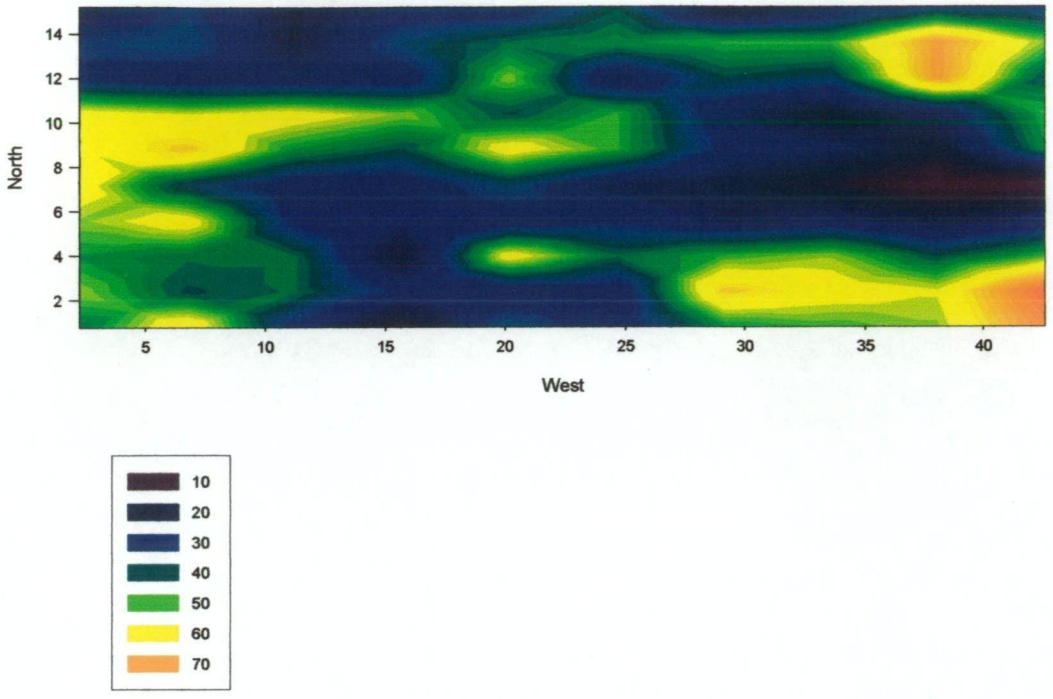

Figure 7.14: *Botrytis cinerea* incidence gradient based on quadrat percentages (at 234 days after sowing). Legend represents percentage of plants infected: 0, 1-10, 11-20% etc.

7.3.2 *Botrytis allii* foci expansion – Infection front

The furthest point from the inoculum source that infected *B. allii* plants were detected was 45 m at 84 days from the time that inoculum was introduced (Figure 7.15). All models were significant at $P < 0.05$, however the Gompertz and logistic models both had high R^2 values of 0.95, compared to the other the linear and exponential models which had R^2 values of 0.88 and 0.87, respectively (Table 7.8).

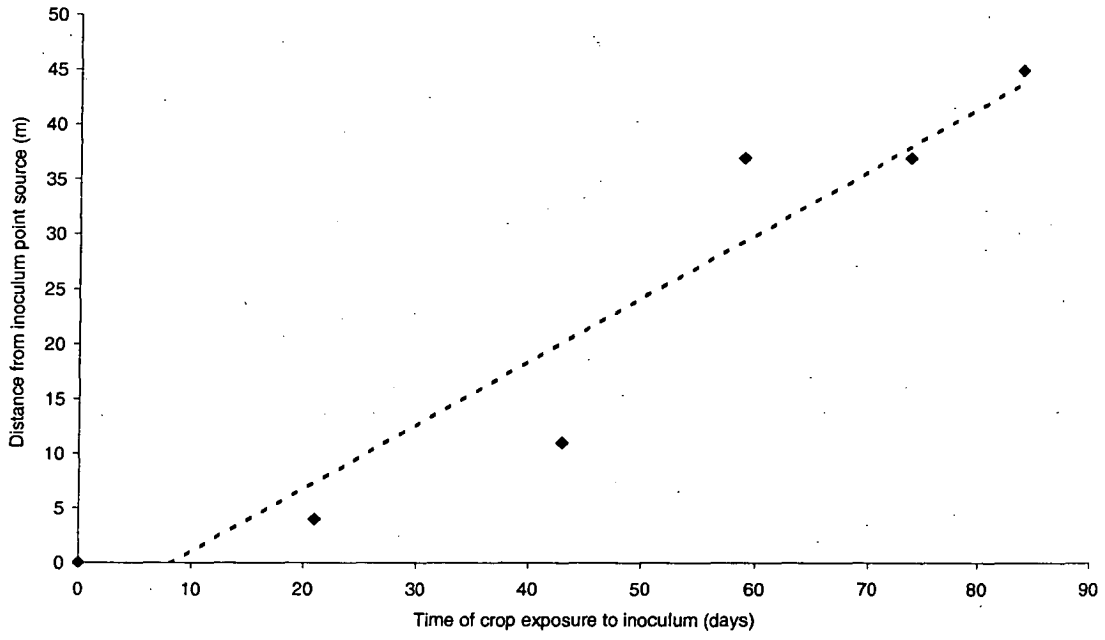

Figure 7.15: Maximum distance between plants infected by *B. allii* and the inoculum source at 0, 21, 43, 59, 74 and 84 days after application of inoculum.

Table 7.8: Regression models fitted to the foci expansion data

Model	F – probability	R^2 values
Linear	0.003	0.88
Exponential	0.022	0.87
Gompertz	0.029	0.95
Logistic	0.030	0.95

7.3.3 Disease progress curves (temporal disease progress)

The cumulative disease progress curves of both *B. allii* and *B. cinerea* were exponential in shape (Figure 7.16 and Figure 7.17, respectively). The disease progress curve of the non-cumulative incidence for *B. allii* and *B. cinerea* was sigmoid in shape. The final incidence of cumulative *B. allii* leaf infection (3.8%) was much closer than the incidence of non-cumulative *B. allii* leaf infection (1.8%) to the bulb rot attributed to *B. allii* (3.6%) in storage.

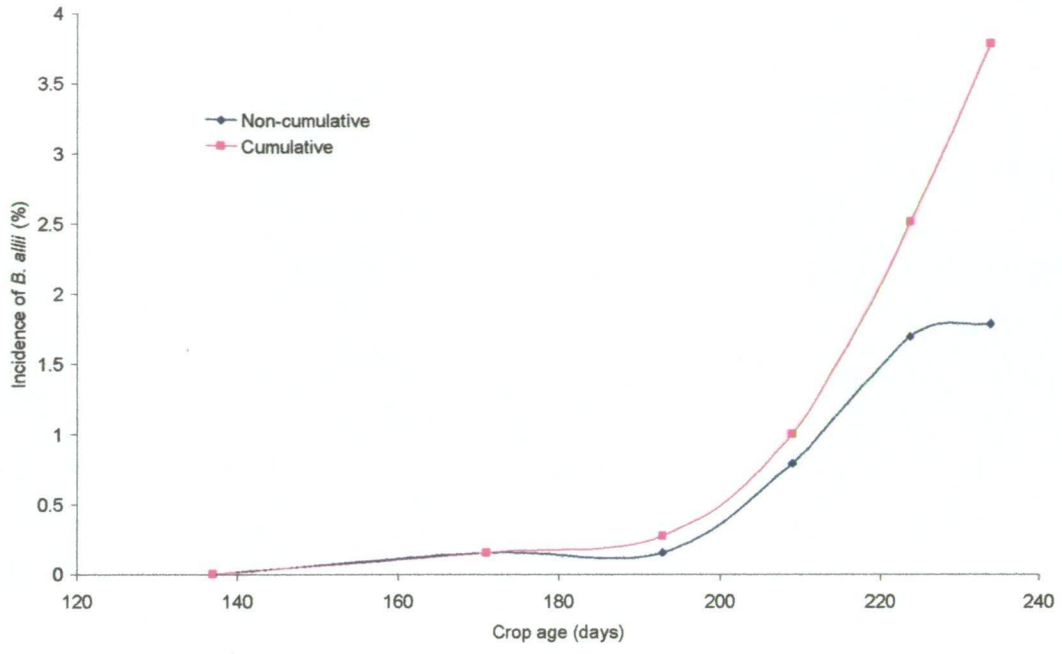

Figure 7.16: *Botrytis allii* disease progress curves

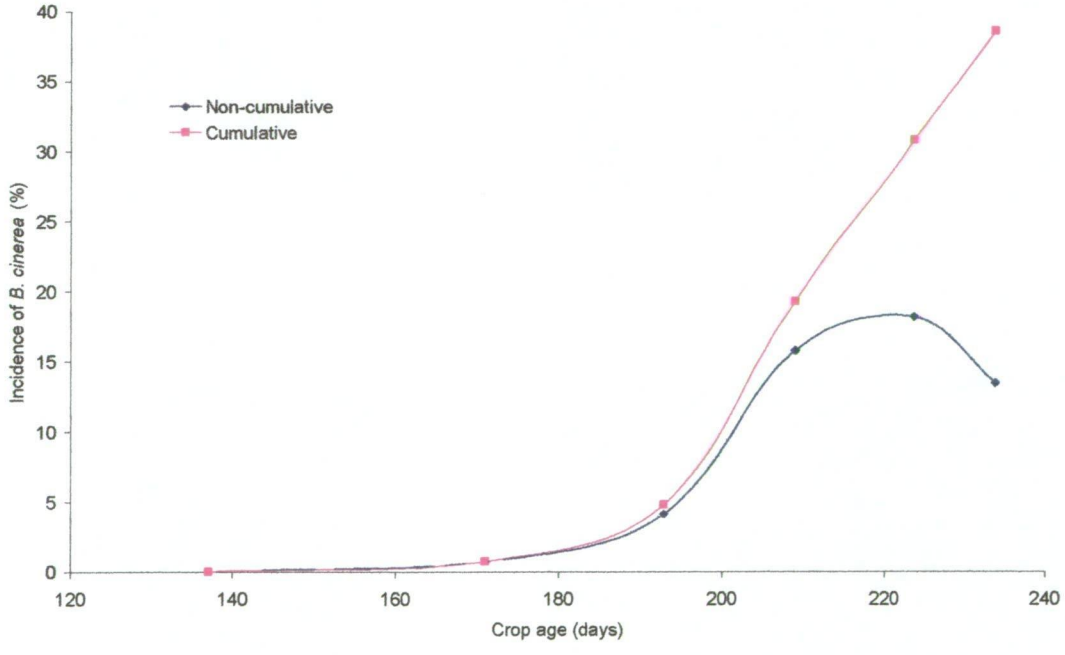

Figure 7.17: *Botrytis cinerea* disease progress curves

Both the cumulative and the non-cumulative *B. allii* disease progress curves fit exponential, logistic and Gompertz models well (Table 7.9), with little difference between the models in R^2, root mean square error and F-probability values.

Table 7.9: Fit of *B. allii* disease progress curves to population growth models

B. allii cumulative incidence		Linear	Exponential	Monomolecular	Logistic	Gompertz
transformed	F	13.38 **	148.75 ****	13.17 **	144.63 ****	69.58 **
	R^2	0.77	0.97	0.77	0.97	0.95
	Root MSE	0.008	0.257	0.008	0.263	0.077
	Intercept	-0.006	-7.030	-0.006	-7.036	-1.990
	rate	0.0004	0.044	0.0004	0.044	0.009
backtransformed	F	13.38 **	502.24 ****	13.07 **	490.44 ****	153.42 ****
	R^2	0.77	0.99	0.77	0.99	0.97
	Root MSE	0.007	0.001	0.007	0.001	0.002
B. allii non-cumulative incidence		Linear	Exponential	Monomolecular	Logistic	Gompertz
transformed	F	15.51 **	50.92 ***	15.4 **	50.65 ***	40.47 ***
	R^2	0.80	0.93	0.79	0.93	0.91
	Root MSE	0.004	0.373	0.004	0.376	0.080
	Intercept	-0.002	-6.978	-0.002	-6.981	-1.967
	rate	0.0002	0.037	0.0002	0.037	0.007
backtransformed	F	15.51 **	29.62 ***	15.34 **	30.01 ***	35.66 ***
	R^2	0.79	0.88	0.79	0.88	0.90
	Root MSE	0.004	0.003	0.004	0.003	0.003

NS = Not significant

* = (P = 0.10)

** = Significant (P = 0.05)

*** = Highly significant (P = 0.01)

**** = Very highly significant (P = 0.001)

MSE = mean square error

The *B. cinerea* cumulative disease progress curve fit the logistic and Gompertz models best, with the Gompertz model being a slightly better fit based on R^2, F-probability and root MSE values than the logistic model. The non-cumulative

progress curve of *B. cinerea* was quite different from the cumulative disease progress curve. Disease incidence initially increased then declined and was fit best by the linear and monomolecular models (Table 7.10).

Table 7.10: Fit of *B. cinerea* disease progress curves to population growth models

B. cinerea cumulative incidence		Linear	Exponential	Monomolecular	Logistic	Gompertz
transformed	F	27.87 ***	112.36 ****	23.06 ***	184.73 ****	315.01 ****
	R^2	0.87	0.97	0.85	0.98	0.99
	Root MSE	0.065	0.489	0.088	0.413	0.100
	Intercept	-0.067	-6.468	-0.088	-6.556	-2.023
	rate	0.005	0.072	0.006	0.078	0.025
backtransformed	F	27.87 ***	24.37 ***	19.05 **	59.88 ***	488.27 ****
	R^2	0.87	0.86	0.83	0.94	0.99
	Root MSE	0.061	0.192	0.076	0.057	0.016
B. cinerea non-cumulative incidence		Linear	Exponential	Monomolecular	Logistic	Gompertz
transformed	F	15.72 **	37.26 ***	14.61 **	38.67 ***	41.99 ***
	R^2	0.80	0.90	0.78	0.91	0.91
	Root MSE	0.040	0.723	0.046	0.738	0.189
	Intercept	-0.017	-6.286	-0.019	-6.305	-1.881
	rate	0.002	0.062	0.002	0.064	0.017
backtransformed	F	15.72 **	3.54 NS	15.54 **	4.61 *	9.67 **
	R^2	0.80	0.47	0.80	0.54	0.71
	Root MSE	0.036	0.105	0.036	0.085	0.051

NS = Not significant (P > 0.1)

* = (P = 0.10)

** = Significant (P = 0.05)

*** = Highly significant (P = 0.01)

**** = Very highly significant (P = 0.001)

MSE = mean square error

7.3.4 Disease gradients

A graph of the *B. allii* disease gradient (Figure 7.18) demonstrated the majority of plants infected by *B. allii* were concentrated within 5 m of the point source.

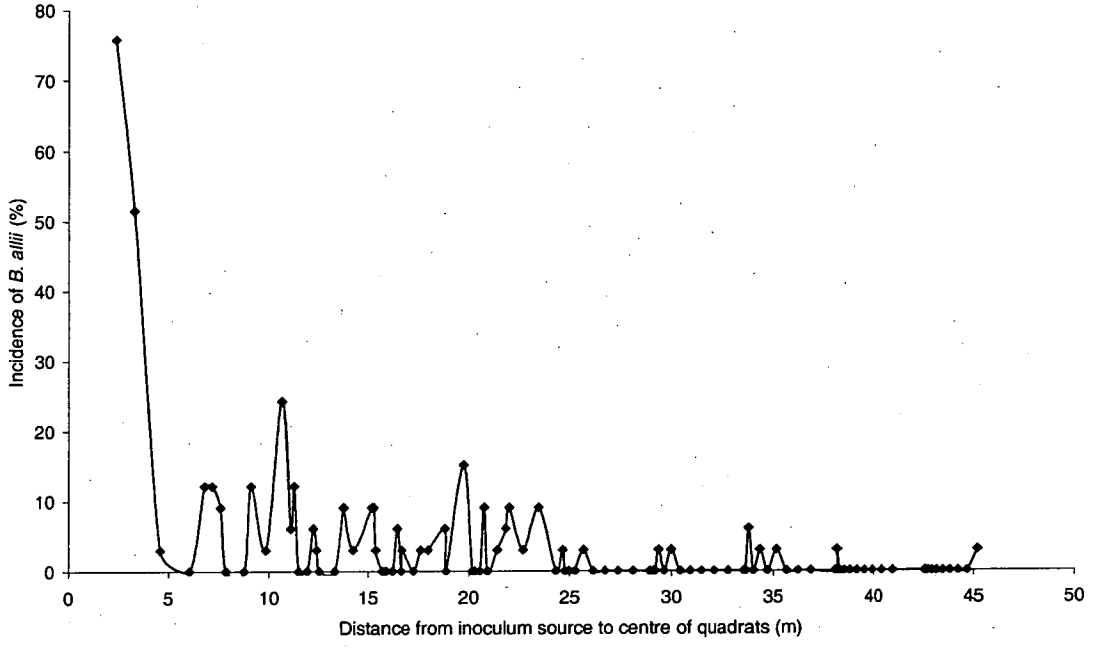

Figure 7.18: Disease gradient of *B. allii*-infected leaves at the 5th cumulative sample, determined on a quadrat basis (distance from inoculum source to the centre of each quadrat)

The plot of disease gradient from inoculum source indicated a similar pattern of *B. allii* spread within rows and between rows (Figure 7.19).

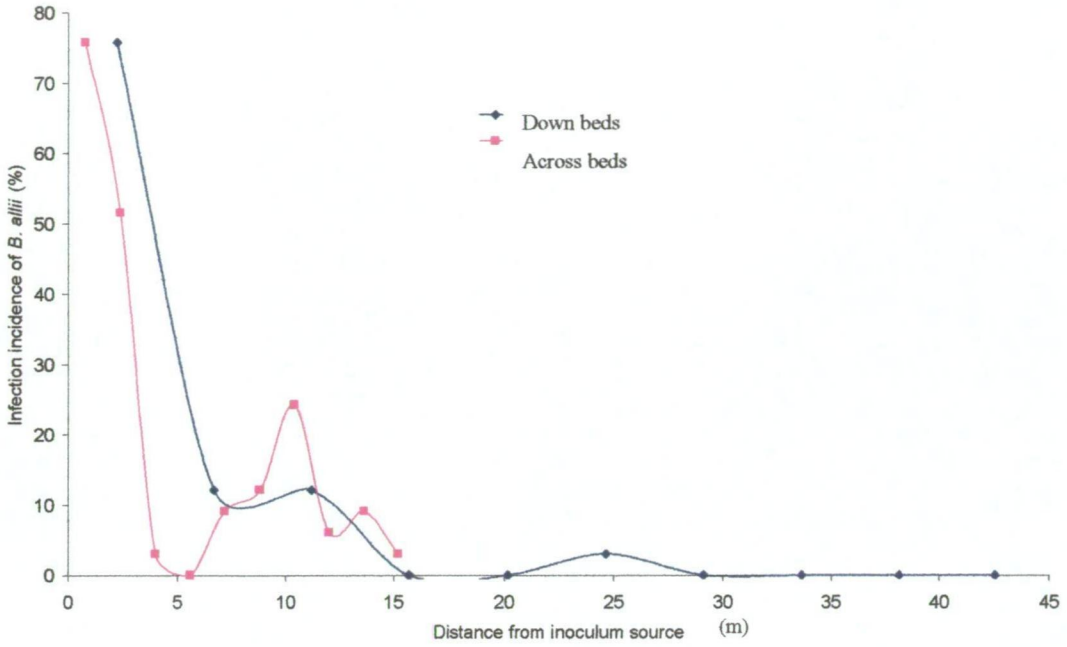

Figure 7.19: Incidence of *B. allii*-infected leaves at distances from the inoculum source within beds and across beds of onions.

The distribution of *B. cinerea* suggested a random disease gradient measured from the inoculum source (Figure 7.20).

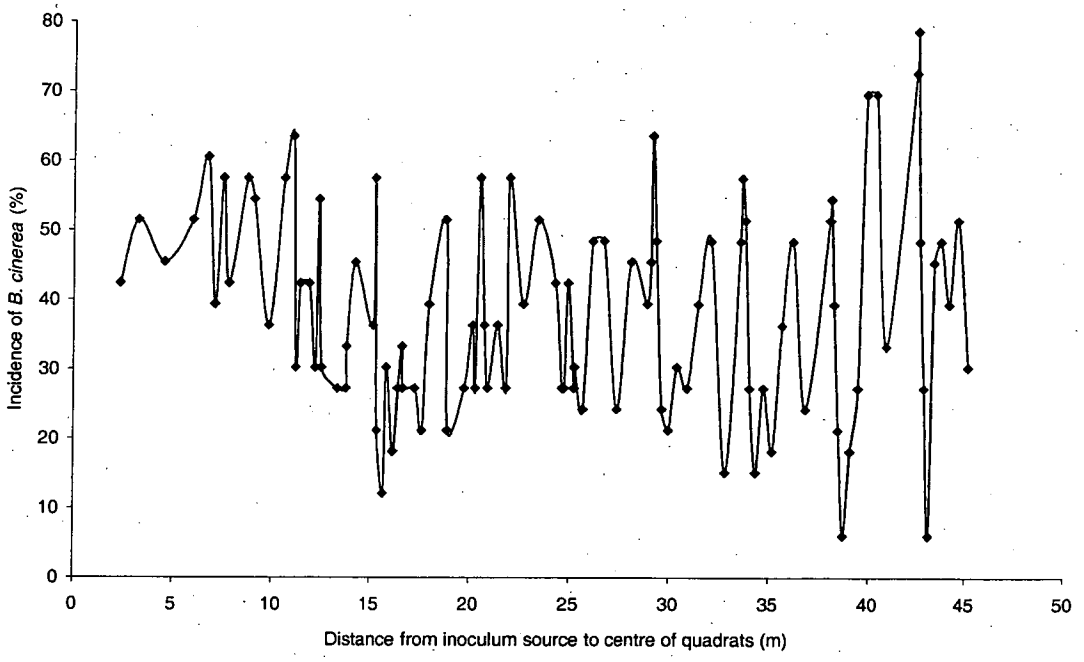

Figure 7.20: Disease gradient of *B. cinerea* at the 5th cumulative sample, determined on a quadrat basis (distance taken from *B. allii* inoculum source to the centre of each quadrat)

The analysis of the gradient of *B. allii* incidence from the inoculum source demonstrated that the exponential model was a better fit to the data than the power law (Table 7.11) as indicated by higher R^2 values at all times of sampling.

Table 7.11: Analysis of *B. allii* infection gradient from the inoculum point source of the field trial (based on cumulative incidence) determined on metre radii from the inoculum source.

	Power law model = A * distance ^{B}			Exponential model = A * exp (-B * distance)		
Time of sampling	R^2	A	B	R^2	A	B
First	0.909	0.078	-0.969	0.950	0.383	1.094
Second	0.959	0.097	-1.081	0.966	0.631	1.535
Third	0.738	0.235	-0.735	0.932	0.719	0.517
Fourth	0.669	0.347	-0.661	0.885	0.961	0.446
Fifth	0.756	0.475	-0.653	0.947	1.217	0.396

F-probability for every model in the above table was significant ($P < 0.001$).

7.3.5 Ordinary Runs Analysis

Ordinary runs analysis of the contiguous quadrats demonstrated that *B. allii*-infected quadrats were distributed non-randomly within ($z = -3.36$; $P = 0.05$) and across beds ($z = -2.45$; $P = 0.05$) (Table 7.12).

Table 7.12: Ordinary runs analysis of *B. allii* cumulative infection at harvest based on 50 quadrats nearest the inoculum source.

	M[1]	N[2]	E(U)[3]	O(U)[4] (within/ across beds)	Z-Statistic[5] (within/across beds)
[6]*Botrytis allii* cumulative infection (50 contiguous quadrats)	50	31	24.56	13/16	-3.36/-2.45

[1] Total number of quadrats

[2] Total number of infected quadrats

[3] Expected number of runs

[4] Observed number of runs

[5] Z-statistic = ([observed number of runs + 0.5 – expected number of runs]/standard deviation), rows treated as contiguous. If Z-statistic is less than –1.64 (P = 0.05), the row of plants has a non-random sequence of infected and healthy quadrats.

[6] Cumulative infection, i.e., a plant found to be infected was assumed to remain so over the duration of the trial. For analysis, rows/beds were treated as contiguous.

7.3.6 Beta Binomial Distribution

Comparisons of LRS values demonstrated that the BBD fitted all the data sets tested significantly (P = 0.05) better than the binomial distribution (Table 7.13). This was supported by an index of dispersion (D) greater than 1 for each data set analysed, which indicated aggregation of infected plants (Madden and Hughes, 1994).

Table 7.13: Analysis of *B. allii* and *B. cinerea* epiphytotics by fitting binomial and beta-binomial distributions.

[1]Data set = sampling time, cumulative or non-cumulative data and number of quadrats in calculations	Index of dispersion (D)	[2]Log ratio test statistic
[3]*B. allii* 5th C 50 quadrats	8.6	139.27
B. cinerea 5th C 50 quadrats	2.3	24.38
B. cinerea 5th C 100 quadrats	3.1	103.42
B. cinerea 4th C 100 quadrats	3.2	110.42
B. cinerea 3rd C 100 quadrats	2.6	66.88
B. cinerea 5th NC 100 quadrats	1.9	25.76
B. cinerea 4th NC 100 quadrats	3.2	108.94
B. cinerea 3rd NC 100 quadrats	2.5	61.20

[1] Analysed if > 5% incidence.

[2] With one degree of freedom, P = 0.05, $\chi^2 = 3.84$.

[3] 5th, 4th, 3rd = data set sampling periods, listed in Table 7.4; C = Cumulative data set, NC = Non-cumulative data set.

7.3.7 SADIE

Analysis by SADIE demonstrated that the incidence of leaf infection by *B. allii* and *B. cinerea,* and the incidence of *B. allii* bulb rot that developed in storage for bulbs sampled from the 50 quadrats closest to the source of inoculum were distributed non-randomly (Table 7.15).

Table 7.14: Spatial analysis of *B. allii* and *B. cinerea* by SADIE

Data set	I_a [1]	P_a [2]
B. allii point data - binary data set	4.712	<0.0002
B. allii - cumulative data 50-quadrats	1.713	0.001
B. cinerea – cumulative data 50-quadrats	1.48	0.0176
B. allii bulb rot data	1.935	<0.0002

[1] Index of aggregation.

[2] Probability that the observed data is no more aggregated than expected from a random permutation of the counts.

The spatial distribution of *B. allii*- and *B. cinerea*-infected leaves and of *Botrytis* bulb rot was significantly aggregated.

The clustering indices for *B. allii* demonstrated a focus close to the inoculum source (Figure 7.21). Areas of relatively large counts close to one another (clusters) are demonstrated in red, while areas of relatively small counts (gaps) are demonstrated in blue. The plot of clustering indices was determined on the 5th cumulative individual plant infection status. A number of other areas of positive aggregation were also demonstrated in the plot. This was viewed as areas of net outflows, i.e., areas from which plants had to be moved during SADIE analysis in order to achieve regularity. The entire binary data set could not be analysed due to limitations in the SADIE program. When the incidence of *B. allii* was analysed on a quadrat basis only, two areas of aggregation were demonstrated (Figure 7.22). There were slight differences in the spatial position of aggregation and depending on the choice of spatial scale, i.e., individual plants (Figure 7.21) versus quadrats of 1.60 m by 4.48 m (Figure 7.22). However, the analyses generally concur that there was significant aggregation of infection close to the inoculum source.

A legend (Table 7.15) is provided for the following plots of clustering indices of spatial distribution and spatial association between populations from SADIE outputs. Confidence limits on clusters are indicated in red and confidence of gaps are indicated in blue at the 90% and 95% confidence intervals, respectively. Where values fall outside of the significance level, ranges are demonstrated in white.

Table 7.15: Key for levels of significance for clustering indices determined by SADIE

	95% confidence of outflows to achieve regularity (aggregation)
	90% confidence of outflows
	No significant flows – random
	90% confidence of inflows to achieve regularity (gaps)
	95% confidence of inflows

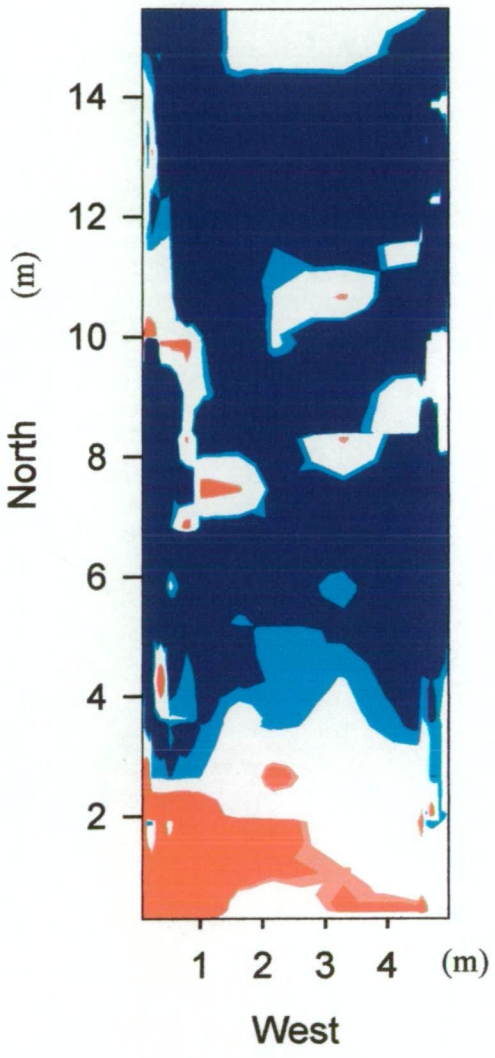

Figure 7.21: Plot of clustering indices of *B. allii* plant infection status at 84 days, based on the infection status of individual plants.

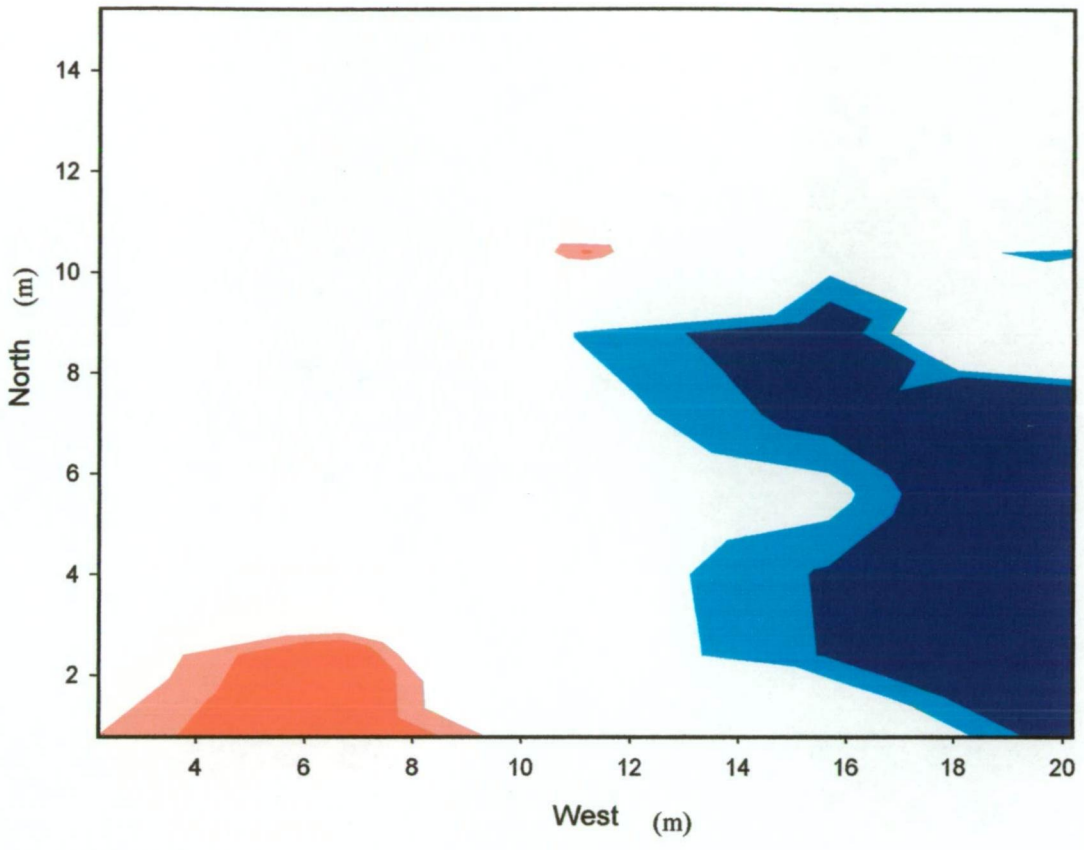

Figure 7.22: *Botrytis allii* clustering indices of plant infection status at 84 days after introduction of inoculum, based on 50 quadrat subset of the field trial

SADIE analysis demonstrated a focus of *B. cinerea* infection around the inoculum source at 234 days after sowing (Figure 7.23).

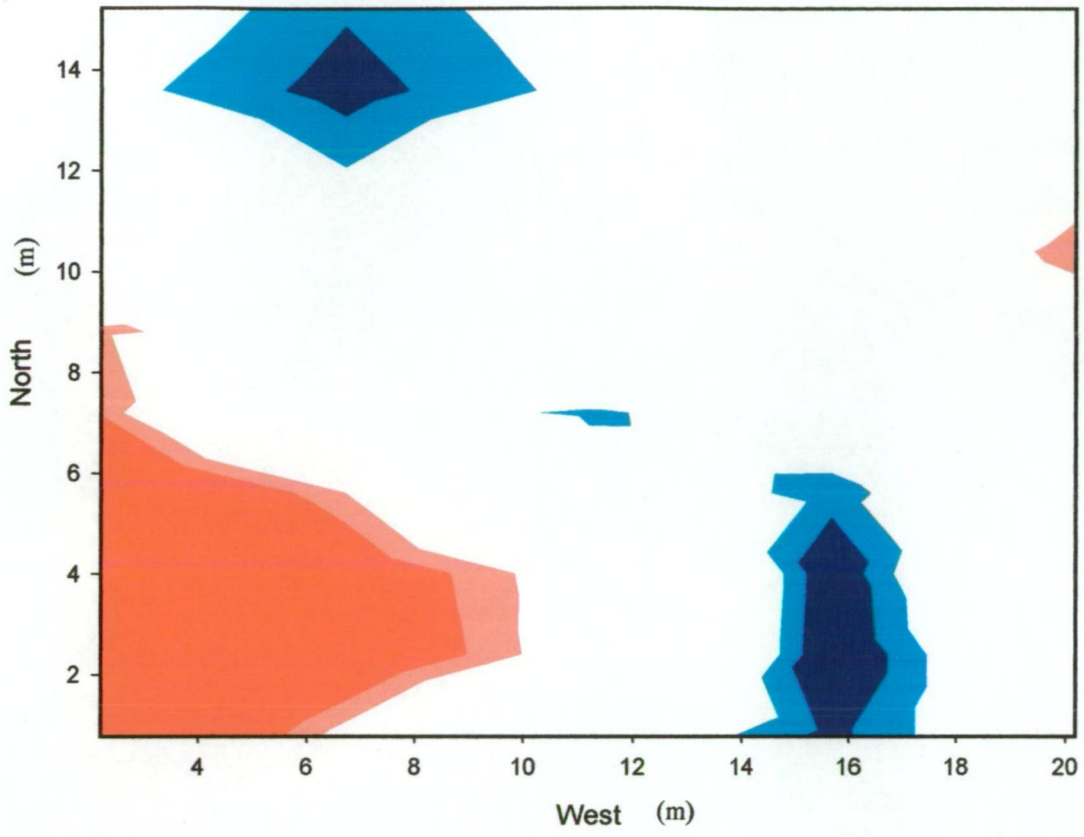

Figure 7.23: *Botrytis cinerea* clustering indices of plant infection status at 234 days after sowing, based on a 50 quadrat subset of the Field trial.

7.3.7.1 Spatial association between *B. allii* and *B. cinerea* (SADIE)

According to SADIE analysis an area of significant ($P < 0.05$) association between the spatial distribution of *B. allii*- and *B. cinerea*-infected plants was located around the point source (Figure 7.24).

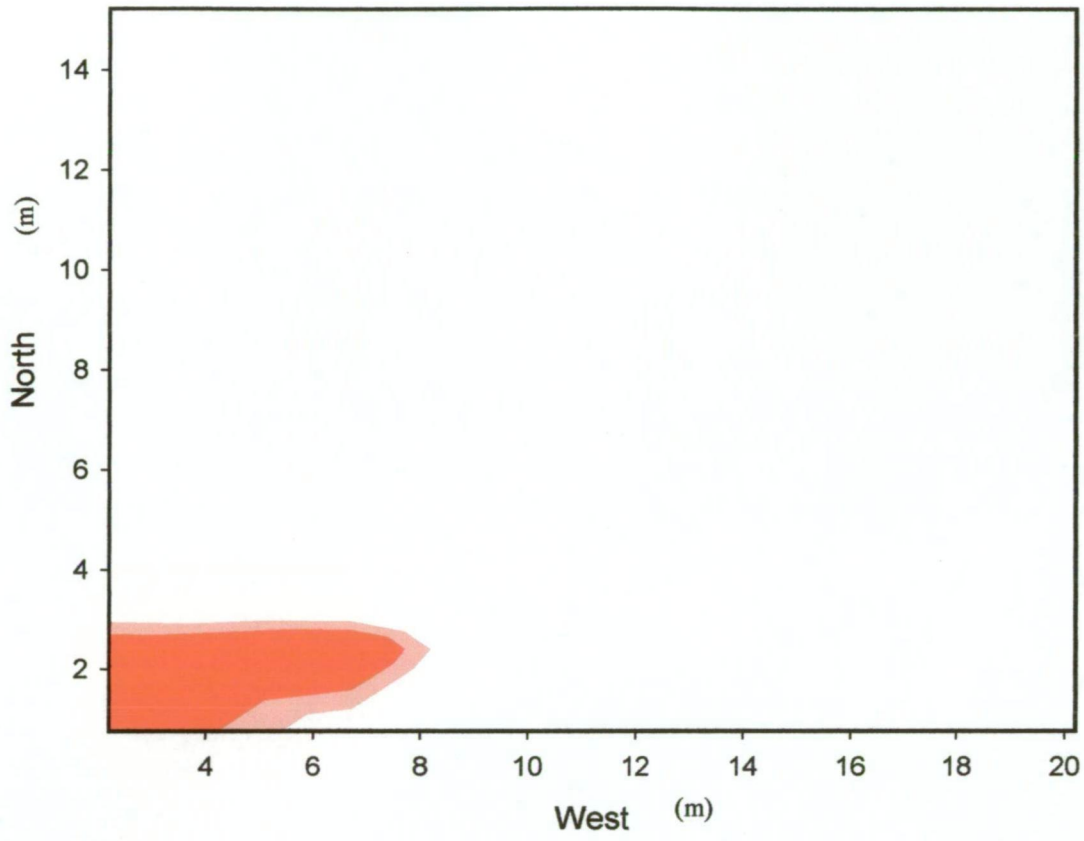

Figure 7.24: Spatial association clustering indices of *B. allii* and *B. cinerea* based on a 50 quadrat subset of the field trial.

7.3.7.2 Spatial association between *B. allii* leaf infection and *Botrytis* bulb rot (SADIE)

High levels of positive correlation occurred between the spatial position of *B. allii* leaf infection at the 5th sampling period and bulb infection in storage for bulbs sampled from some areas of the plot, particularly samples collected close to the inoculum source where a higher incidence of *B. allii* leaf infection and a higher incidence of *B. allii* bulb infection were found relative to the rest of the area evaluated (Figure 7.25). The measure of overall association X = 0.5049, (Dutilleul adjusted) had a probability level of 0.0004. The plot of spatial association demonstrated that there were no areas

of negative association between *B. allii* leaf infection and *Botrytis* bulb rot (Figure 7.25).

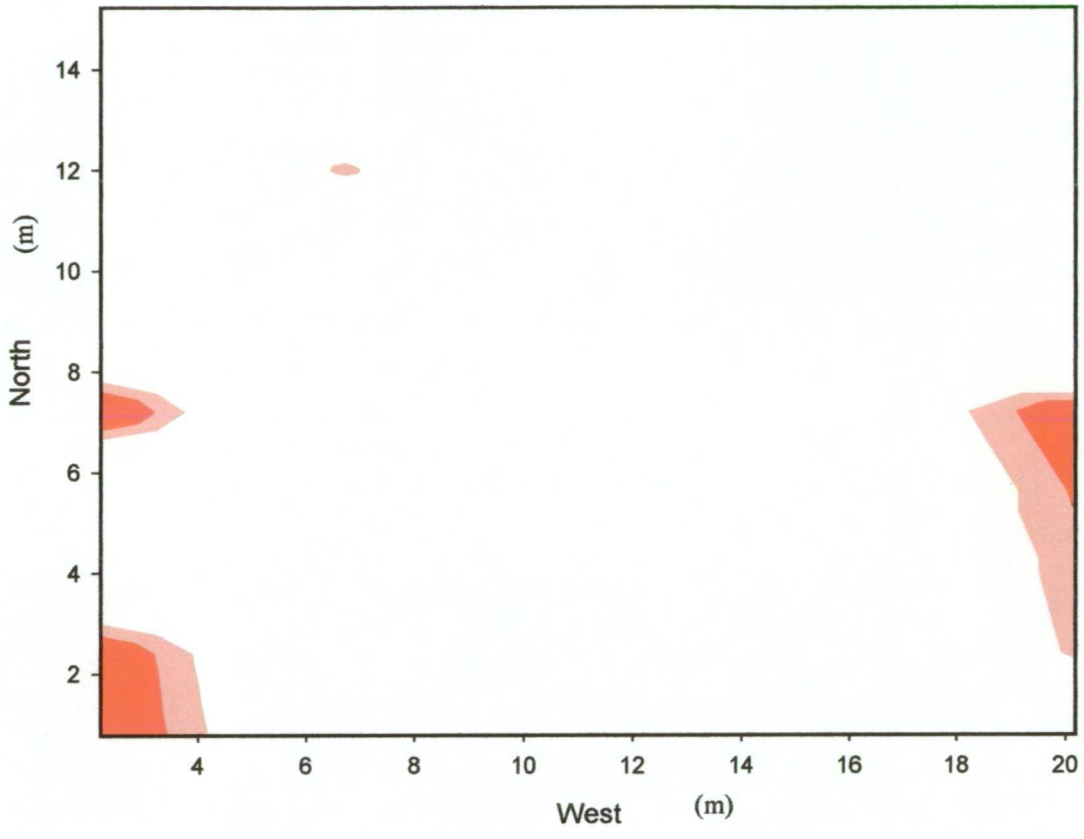

Figure 7.25: Spatial association clustering indices of *B. allii* leaf infection and *Botrytis* bulb rot based on 50 quadrat subset of the field trial.

7.3.8 Radial correlation analysis

Radial correlation analysis demonstrated significant aggregation between *B. allii*-infected plants both within rows and radially to a distance of 16 m, ($P < 0.05$) (Figure 7.26). Radial correlation analysis was not performed across rows due to the low number of plant pair counts in this direction.

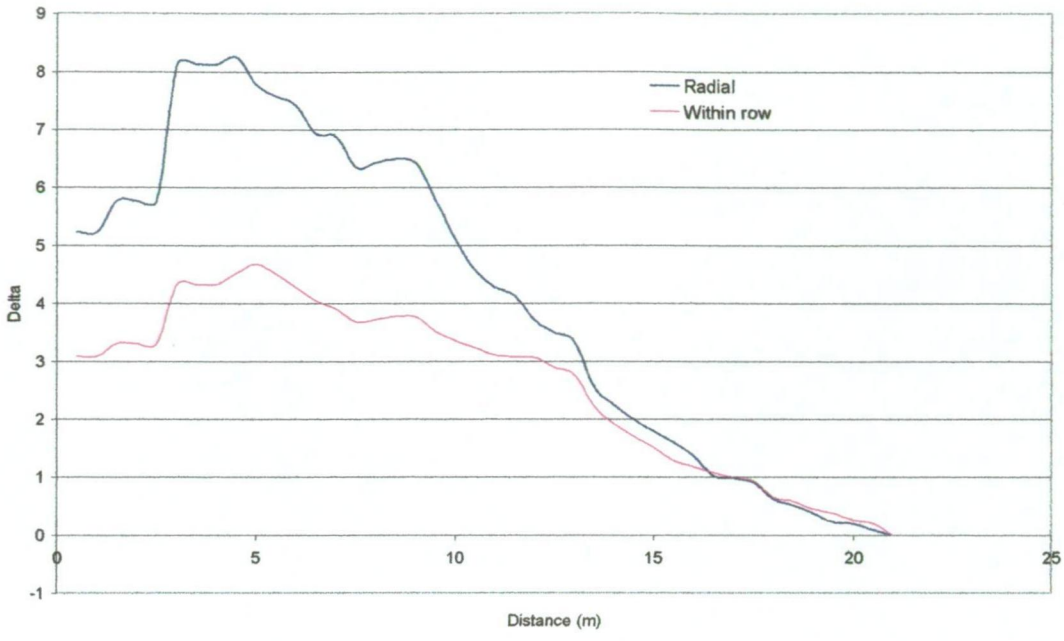

Figure 7.26: Normalised density of *B. allii*-infected plants as a function of radial distance from an infected plant, based on spatial position of *B. allii*-infected plants 84 days after inoculation of the trial (values > 1 are significant at P < 0.05)

Radial correlation analysis carried out without the *B. allii*-infected plant pairs in the first two beds demonstrated aggregation within rows was almost significant at $P < 0.05$, up to a distance of 5 m (Figure 7.27). The level of confidence reached a significance of $P < 0.05$ just after 5 m and then declined again. Evaluating the infected plant pairs radially did not detect any significant ($P < 0.05$) aggregation (delta > 1) of infected plants at any distance from the source of inoculum.

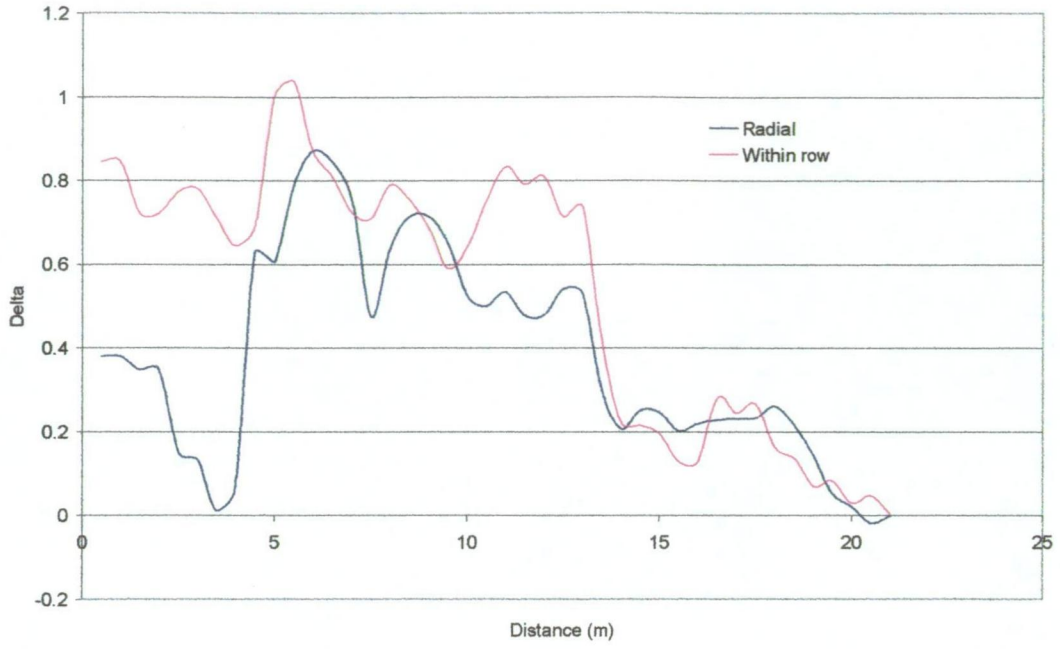

Figure 7.27: Normalised density of *B. allii*-infected plants as a function of radial distance from an infected plant. Analysis performed without the first 2 beds, based on spatial position of *B. allii*-infected plants 84 days after inoculation of trial (values > 1 are significant at P<0.05).

A Kolmogorov-Smirnov (KS) statistic was not calculated, as the incidence of *B. allii*-infected plants were too low.

7.4 Discussion

7.4.1 Disease maps

The maps of spatial distribution of *B. allii* indicated that spread most likely occurred from the source of inoculum, with the majority of subsequent spread close to the inoculum source. Gradual spread of *B. allii* occurred during the field trial, taking *B. allii* 84 days to reach 45 m within the extent of the sample area. The onion bulb inoculum source was not expected to contribute to the epidemic past approximately 7 days after placement, as the bulbs decayed rapidly after being placed in the field.

In contrast, the maps of spatial distribution of *B. cinerea* infection demonstrated an apparent random appearance of infection foci, most likely the result of windborne spores coming from external sources. Alternatively, the distribution of *B. cinerea* may have resulted from its random occurrence on organic matter throughout the trial site and subsequent spread to onion plants. An area of higher incidence of *B. cinerea* infection did occur in close proximity to the inoculum source, indicating the inoculum source may have contributed to the prevalence of *B. cinerea* in that part of the field.

7.4.2 Foci expansion – infection front

The infection front expansion was described well by the Gompertz and logistic models, suggesting that the infection front did not expand with a constant velocity. This finding opposes that of Minogue and Fry (1983a,b), who stated that under constant conditions, the population is expected to spread as a wave at a constant velocity. Van den Bosch *et al.* (1988) also reported that once an initial phase of infection focus build up has occurred, the front of the focus expands radially at a constant velocity. The term 'front' is defined as the part of the disease profile that is in the pre-saturation phase or the foremost tail of the profile (van den Bosch *et al.*, 1988). Perhaps, the velocity of the *B. allii* infection front was affected by environmental conditions, progressing during favourable conditions and slowing during unfavourable conditions, such as lack drier periods. Further work will be required to investigate the velocity of the *B. allii* infection front. The *B. allii* infection front suggested that *B. allii* spread predominantly over short distances, i.e., inoculum infected a plant, which produced inoculum that spread to another nearby plant etc.

7.4.3 Disease progress curves (temporal disease progress)

The exponential, logistic and Gompertz models described the increase in incidence of *B. allii*-infected plants well, with back transformed R^2 values of 0.99, 0.99 and 0.97, respectively. There were few differences between the exponential model, the logistic model and the Gompertz model when disease incidence was low. As the epiphytotic was only starting, there were no limitations placed on the pathogen in terms of the number of plants remaining to be infected. If the epiphytotic had progressed further, the models which describe the disease progress curve would change. Models which take into account the reduction in number of suitable hosts with time will fit the disease progress curve better, such as the Gompertz, logistic and monomolecular models.

Most of the models had relatively high R^2 values (> 0.7), particularly after back-transformation. Campbell and Madden (1990) stated that because of the cumulative nature of disease progress curves, high R^2 values are often found, even if an inappropriate model is used. Thus care must be used when interpreting the results. Analysis of disease progress curves is often used to describe epiphytotics as this provides an insight into the mechanics of spread of pathogens.

The disease progress curves of *B. cinerea* infection did not increase much after the third sampling at 209 days after planting. However, the incidence of *B. allii* did not begin to plateau until the fourth sampling at 224 days after planting. The difference in shape of disease progress curves between the two species may be associated with the nature of the pathogenicity of the two species. *Botrytis allii* is more pathogenic on onion than *B. cinerea* (Stewart and Mansfield, 1984; Hancock and Lorbeer, 1963). As

the foliage became more susceptible to other plant pathogens or saprophytes through breakdown of plant defences, there was more competition for resources, and the more pathogenic *Botrytis* species may have prevailed for longer. The number of healthy plants may have become a limiting factor for *B. cinerea* earlier than for *B. allii,* due to a much higher incidence of the former. Furthermore the source of inoculum of *B. cinerea* may have been much greater than that of *B. allii* and more spatially widespread leading to a steeper disease progress curve.

Botrytis allii and *B. cinerea* appear to be late-season pathogens, with infection rapidly increasing late in crop maturity. Late in the crop life being defined as after about 200 days since planting, until lifting of the plants, which occurred 35 days later. The rapid increase in both *Botrytis* spp. also appears to be correlated to the tops down stage of the onion crop, where the canopy begins to fall over with maturity. *Botrytis cinerea* appeared not to be source-specific (within the crop), and was probably present in the field before it was detected at a low incidence initially. Foci of *B. cinerea* appeared to originate at random, which is characteristic of a pathogen entering aerially from a distant source (Gregory, 1968). *Botrytis allii* was source-specific, not infecting the crop until the inoculum source was introduced, and then spreading from the inoculum source into the crop. *Botrytis squamosa* has also been reported as a mid- to late-season pathogen (Small, 1970; Swanton, 1977), due to the influence of senescing onion tissue on infection once bulb initiation has begun (Alderman and Lacy, 1984b). Foliage senescence provides leaf material that is easier for such fungi to colonise due to breakdown of the leaf tissue and plant resistance mechanisms. Alderman and Lacy (1984a) suggested that the greatest access to bulb scales occurs after the onions lodge, when leaves rapidly collapse and die. *Botrytis allii* and *B. cinerea* disease progress

curves may be similar to that of *B. squamosa*, as all of these fungi require senescent tissue to sporulate.

The cumulative disease progress curve demonstrated the change in new infections that occurred through the season, as once a plant was infected it remained recorded as infected, even though the infection of the plant may have been removed through the sampling of the infected leaf. When a pathogen is spreading to plants that have not been infected, the cumulative disease progress curve should increase more steeply than the non-cumulative disease progress curve. The non-cumulative disease progress curve is a better measure of the epidemic at each sample time, as the shape of cumulative disease progress curve is influenced by the results of previous sample periods.

The trial illustrated that the incidence of *B. allii*-infected plants could increase in an exponential manner. Therefore, very small amounts of inoculum can become economically-damaging within a short period of time. The best way to control polycyclic diseases such as that caused by *B. allii* is to 1) reduce the amount of initial inoculum; 2) reduce the time available for the epiphytotic to develop; 3) reduce the rate of disease increase (Campbell and Madden, 1990; Brown *et al.*, 1997). Best management practices should be practised, to control *B. allii* including, reducing initial inoculum by establishing crops with seed that has been tested for *B. allii* and has an acceptable level, that the seed is treated to manage any seed infection present and to remove inoculum sources such as volunteers and cull piles. The rate of *B. allii* increase can be managed by implementing the current fungicide regime (Section 9.1.2). Other strategies to reduce the rate of infection may be implemented in the

future such as irrigation practices. To reduce the time available for the epiphytotic to develop, early-maturing cultivars can be used. This may be possible for *B. allii* management, however this will require experiments to determine the benefits (Campbell and Madden, 1990; Brown *et al.*, 1997).

7.4.4 Disease gradients

The *B. allii* plant infection gradient was explained better by the exponential model than the power law model, suggesting the epiphytotic was predominantly driven by short distance movements of spores, most likely as a result of splash dispersal. Although wind-dispersal of *B. allii* spores was not excluded, it appears that short-range spread was very important in this *B. allii* epiphytotic. Maude and Presly (1977a,b) assumed that *B. allii* was capable of aerial spread. In one trial they indicated the fungus had spread from an infected plot to an isolated plot 270 m away (Maude and Presly, 1977a). It is likely that *B. allii* is also capable of long-range aerial dispersal. However, demonstrating the amount and effectiveness of long-range aerial dispersal is difficult. Future studies utilising genetic markers could be used to examine long-range spread of this pathogen. For example, (Hovmoller *et al.* 2002) demonstrated a single clonal population of the biotrophic fungus *Puccinia striiformis* f.sp. tritici over the UK, France, Germany and Denmark up to 1700 km apart.

Botrytis conidia often detach from the conidiophore in clusters. This results in a greater settling speed and higher rates of deposition near the source than for single conidia (Harrison and Lowe, 1987). Although single spores of *B. allii* were observed in the spore traps (Chapter 6), clusters of spores may have dispersed also, resulting in steep disease gradients. Wind dispersal of conidia can also result in steep disease

gradients if some conidia are deposited close to the source while others escape from the canopy and are carried away from the crop (Ferrandino, 1993).

Stewart and Mansfield (1984) reported a loss of virulence of *B. allii* spores associated with low concentrations of the spores. They suggested that this may be caused by the loss of endogenous nutrients into surrounding water droplets, which may decrease rates of enzyme synthesis during the critical early stages of growth of infection hyphae. This may have prevented the invading fungus from killing epidermal cells before the plant cells produced resistant reaction-material and prevented infection. If low numbers of conidia are deposited by wind dispersal, the spores may be unsuccessful in colonisation of plant tissue. However, if the spores are dispersed in high concentrations by splashing rain droplets (even as clusters), they may be more likely to infect the plant successfully. Free water from the splash droplet is also likely to assist in spore germination and plant infection (Chapter 5). Hence, splash dispersal may have advantage over other means of conidial dispersal which result in lower concentrations of conidia. Interestingly, Kritzman *et al.* (1980) reported that at high concentrations of *B. allii* conidia germination is inhibited, possibly through the release of an inhibitory compound. This inhibition may be overcome by stimulation from the plant on the leaf surface. This aspect of *B. allii* biology requires further study.

7.4.5 Spatial patterns of *B. allii* epiphytotics

All the spatial analysis techniques used in this study suggested the spatial distribution of *B. allii* was aggregated. This was suggested visually by the disease maps and spatial depiction of the disease gradients, which clearly demonstrated an aggregation

of infected plants around the inoculum source and subsequent development of secondary, clustered foci.

The apparent random spatial pattern of *B. cinerea*-infected plants early in the growth of the crop suggested this fungus entered the crop randomly, with subsequent short-range spread from the infected plants causing aggregated spatial patterns. Kohl *et al.* (1995) noted that a background incidence of airborne conidia of *B. cinerea* may always be present in an onion crop even when no *B. cinerea* infection is detected in the onion crop. However, following epiphytotic initiation, the influence of inoculum from outside the crop may become less important as secondary spread occurs within the crop (Kohl *et al.*, 1995). *Botrytis allii* might be expected to behave in a similar manner to *B. cinerea* in situations where it is spread by wind over long distances.

A significantly positive association between *B. allii* and *B. cinerea* occurred in the area close to the inoculum source. This suggested the *B. allii* inoculum source may also have provided some *B. cinerea* inoculum. Alternatively, the area of positive association of *B. allii* and *B. cinerea* may have been coincidental, as it was apparent when the maps of *Botrytis* incidence were observed that there were a number of *B. cinerea* foci located throughout the paddock, and only one focus of *B. allii*.

Spatial analyses in this study using several techniques demonstrated the aggregated spatial pattern of *B. allii*- and *B. cinerea*-infected plants.

7.4.6 Correlation between the incidence of *B. allii* in leaves and bulbs

The cumulative incidence of *B. allii* detected in the leaves at 84 days after introduction of inoculum (3.8%) was similar to the incidence of bulb rot in storage (3.6%). However, the non-cumulative final incidence of *B. allii* in leaf samples (1.8%) underestimated the incidence of *Botrytis* bulb rot in storage. Once a leaf becomes infected and it senesces, *B. allii* can grow down the leaf and into the neck of the bulb. Thus, a leaf sample taken at any time during the crop life may not be an accurate indication of the final incidence of *Botrytis* bulb rot, as new infections may occur subsequently or leaves that were infected may have senesced and may have already contributed to bulb infection. These results suggest that *B. allii* does not move systemically throughout the whole plant after infection. Tichelaar (1967) considered *B. allii* to be quiescent in the green leaf epidermis, only invading the leaf mesophyll tissue when the leaf senesced. Bochow and El Mosallamy (1979) also reported latent infections of green leaf and juvenile meristematic tissue. Senescent tissue was found to be infected more often by Bochow and El Mossalamy (1979). However, Maude and Presly (1977a) demonstrated that *B. allii* can move within the green leaf. They suggested two types of plant infection: 1) continuous infection throughout the whole plant, which originates from infected seed and develops from the base of each successive leaf that develops; and 2) the more common discontinuous form of infection of leaf tips with subsequent growth of the fungus down the leaves (Maude and Presly, 1977a). The latter type of infection occurred in this study, the percentage of cumulative *B. allii* infected leaves (3.8%) found was very similar to the final incidence of neck rot (3.6%), however the incidence of non-cumulative *B. allii* infected leaves (1.8%) was lower than the level of neck rot, indicating that infections were discontinuous throughout the onion plant. As the onions cured the *B. allii*

infections would have progressed down the leaves and into the neck of the onion bulbs.

Chapter 8

8 Effect of cultural practices on the *Botrytis allii* epiphytotic (Field trial 2)

8.1 Introduction

The role of seed infection and agronomic factors in epiphytotics of *B. allii* neck rot are not well understood. Maude (1983) noted that during dry growing seasons there was almost a one-to-one direct linear relationship between the percentage of *B. allii* infection in seeds sown and the percentage of subsequent bulb neck rot. In wet, humid growing seasons the proportion of bulbs with neck rot was proportionally greater than the incidence of *B. allii* in seed (Maude, 1983). Stewart and Franicevic (1994) reported that occasionally the incidence of *B. allii* detected in seedlings was lower than that detected in seeds. Tichelaar (1967) and Maude and Presly (1977b) reported that the use of a benomyl seed dressing greatly reduced the incidence of resultant bulb rot in crops planted with infected seed lots. The experiments described in this chapter were aimed at gaining a better understanding of the seed-to-seedling transmission of *B. allii* and the effect of nitrogen and irrigation on the incidence of *B. allii* infection in onion crops.

8.2 Materials and methods

8.2.1 Seed test

A seed lot with a high incidence of *B. allii* and a "clean" seed lot were used in the field experiment. The incidence of *B. allii* "infected" and "clean" seed lot was ascertained using the methods described in Appendix 12.4. One thousand seeds were tested per lot, with five seeds per Petri plate.

8.2.2 Field trial preparation

A soil sample was collected from the site of Field trial 2 in July of 2000 by Field Fresh Tasmania Pty. Ltd., and analysed at Allison Laboratories in Hobart. The results are given in Appendix 12.10. The previous crop grown in the field was pasture (ryegrass/clover). The field trial area was fertilised with 6 t/ha of lime and 1300 kg/ha of 3-15-13-1 (N 40: P 200: K 170: S 13 kg/ha), plus zinc (3.5 kg/ha), applied prior to sowing. Triple superphosphate was drilled with the seed at a rate of 125 kg/ha, except on the inoculum source comprised of a bed of onion sown with an infected seed lot (Figure 8.1). Further nitrogen treatments were applied as described in Section 8.2.7. The field trial was sown at 3.3 kg seed/ha on the 29/8/00 using an Accord precision drill, with 9 rows of onions per bed. Seed with a high incidence of *B. allii* was left untreated with fungicides and was sown into the first bed (which will be referred to as the 'infection bed'). The rest of the trial was sown with the 'clean' seed lot 9-C-175, treated with the standard onion seed treatment of a Benlate®/Thiram® dust and wallpaper glue. The total area sown was 80 m in length, consisting of 21 beds with 9 rows per bed. Beds were sown in an east-west orientation. Bed width was 1 m and beds were separated between by 0.6 m alleys. Pesticides used during the trial are

listed in Table 8.1. No fungicides were applied during the crop season as they may have restricted *Botrytis* development. No downy mildew (*Peronospora destructor*) was observed.

Table 8.1: Herbicides and insecticides used during Field trial 2.

Date	Pesticide
Prior to cultivating	Roundup[1]
20/9/00	Sprayseed[2] 2.4 L/ha + Stomp[3] 1 L/ha
3/10/00	Lorsban[4] 700 ml/ha (insecticide for cutworm)
20/10/00	Stomp 2.0 L/ha
Date undetermined	Verdict[5] 520 200 ml/ha + 100 ml Activator/100 L
"	Tramat[6] 800 ml/ha
"	Totril[7] 500 ml/ha
"	Tribunal[8] 400 g/ha
4/12/00	Totril 750 ml/ha + Bladex[9] 750 ml/ha
6/1/01	Totril 500 ml/ha + Bladex 500 ml/ha

[1]Glyphosate, [2]-, [3]Pendimethalin, [4]Chlorpyrifos, [5]Haloxyfop-methyl (unstated stereochemistry), [6]Ethofumesate, [7]Ioxynil, [8]Methabenzthiazuron, [9]Cyanazine.

8.2.3 Trial layout and design

The trial layout was set-up so that there were four experiments. 1) the nitrogen trial carried out under high irrigation practice, 2) the nitrogen trial carried out under low irrigation practice, 3) the spatial analysis trial carried out under high irrigation practice and 4) the spatial analysis trial carried out under low irrigation practice (Figure 8.1). On the northern side of the irrigation run, nitrogen treatments were applied to the beds receiving each of the two irrigation treatments (Section 8.2.7). The southern side was used for spatial assessment under the two irrigation treatments (Section 8.2.8).

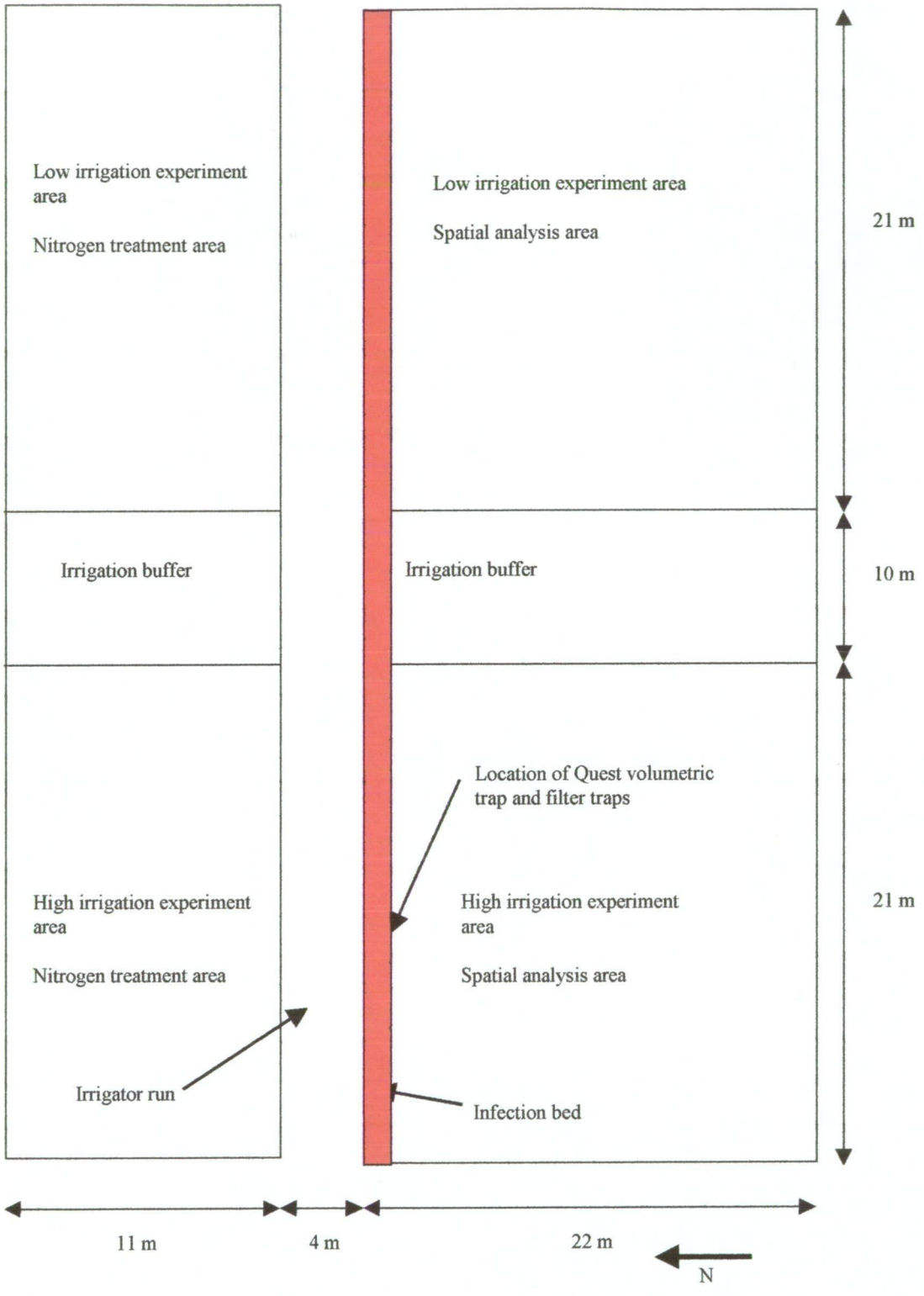

Figure 8.1: Field trial 2 layout: Treatments are described in the following sections. In the spatial analysis area, the first bed (highlighted in red) was the inoculum/infection bed. Within the nitrogen treatment area, the bed closest to the spatial analysis area was Bed 1, with Bed 7 located at the other side of the nitrogen treatment area. Within the spatial analysis area, the infection bed was Bed 1, and the furthest bed from this was Bed 14.

8.2.4 Application of inoculum

A low incidence of *B. allii* was detected in samples taken from the infection bed early after planting (Table 8.2). Therefore, the inoculum source bed was reinfected with *B. allii* on the 5/1/02 (Table 8.3), to increase the probability that surrounding plants would become infected. This application was repeated on the 14/1/02 (Table 8.3). A spore suspension was also applied directly to the nitrogen treatment Bed 7 on 13/02/01.

Table 8.2: Dates and numbers of leaf samples taken from the infection bed and examined for infection before establishing *B. allii* infection on 14/1/01.

Date	Number of leaves sampled from infection bed
22/11/00	400
4/12/00	40 (whole plants)
19/12/00	50
4/1/01	200
8/1/01	50

Inoculum (isolate C3 of *B. allii*) was prepared by cutting artificially-inoculated onions in half (Appendix 12.3) and leaving the cut bulbs for one week to promote sporulation. Spore suspensions were made by placing each onion half into a jar and shaking the onion in the jar with 200 ml of water and one drop of Tween 80. Four onions were used to produce each spore suspension. The suspension was passed through a 50 µm sieve to remove material that may have clogged the knapsack sprayer. A sample of the spore suspension was taken to count spores with a haemocytometer. The spore suspension was then made up to 4 L in a knapsack sprayer. The suspension was applied through a plastic flat fan (Hardi) nozzle. The output of the knapsack sprayer was 340 ml/min at 40 psi. The ground speed was regulated by walking with an electronic metronome at 40 steps/min.

The spore suspensions were applied on the morning of 5/1/01 to a 58 m section of the infection bed (Figure 8.1), which included 10 m of irrigation buffer and 24 metres in each of the two irrigation experiments. Conditions were overcast with no wind, and leaves were wet from overnight rain. The first spore suspension was applied at 7.30 am walking down the bed (outside Bed 1). The second was applied at 9.00 am walking up the bed (between Beds 1 and 2). Approximately 25 mm of water was applied to the trial with a travelling gun-type irrigator (described below) on 5/1/00 to maintain leaf wetness and promote infection. The first spore suspension 1, was made from 610 ml of 7.1×10^5 spores/ml, diluted to 1.1×10^5 spores/ml of which 1570 ml was applied. Suspension 2 was made from 670 ml of 4.45×10^5 spores/ml, diluted to 3.0×10^5 spores/ml of which 1570 ml was applied to the infection bed. Therefore, the combined number of spores applied to the infection bed on the 5/1/00 was 4.71×10^8 spores applied to 58 m^2 = 8.12×10^6 spores/m^2. Assuming 90 plants/m^2 approximately 9×10^4 spores were applied to each plant.

Another application of spores was made on the 14/1/01 at 8.30 pm, as infected leaves were not detected in a sample collected after the first two applications of inoculum (Table 8.3). Suspension 3 was made from 590 ml of 12.4×10^5 spores/ml, diluted to 1.8×10^5 spores/ml of which 2840 ml was applied to the infection bed in two spray passes. Therefore, 5.2×10^8 spores were applied to 58 m^2 of bed length or 9×10^6 spores/m^2. Assuming 90 plants/m^2 this equated to approximately 1×10^5 spores/plant. Inoculum was applied during overcast conditions. A check was also made for germination of the spores in suspension as a measure of viability. Spores were sprayed onto 1% water agar in three Petri plates and observed for germination 12 hours later with a microscope.

8.2.5 Events of field trial

The schedule of field trial events relative to the crop age, as determined by days after sowing, is described in Table 8.3.

Table 8.3: Schedule of events of Field trial 2, with days after sowing and days after application of inoculum. Dates are included for all major events regarding treatment applications and trial assessments.

Date	DAS[A] (DAI[B])	Event
29/8/00	0	Crop sown
22/11/00	85	1st sample (spatial area + N treatments)
4/12/00	97	40 plant samples sampled from irrigation buffer of infection bed, whole plants were sampled to look for internal infection vs. leaf infection
5/12/00	98	Urea application to all plots
16/12/00	109	Applied additional urea to N+ plots
19/12/00	112	Samples of 50 leaves taken from spatial area Beds 1, 2 and 3 and from N treatment Bed 1
27/12/00	120	Applied additional urea to N+ plots
4/1/01	128	Leaf samples collected (10 leaves per quadrat from spatial analysis area Beds 1 and 2 and from N treatment Bed 1)
5/1/01	129	Spore suspension applied
8/1/01	132	50-leaf sample collected from irrigation buffer, infection Bed 1,2,3 and N treatment Bed 1
14/1/01	138 (0)	Spore suspension reapplied
15/1/01	139 (1)	2nd sampling of trial (spatial area + N treatment area)
18/1/01	142 (4)	Applied urea to N+ plots
31/1/01	155 (17)	3rd sampling of trial (spatial area + N treatment area)
1/2/01	156 (18)	Leaf samples collected for nitrate analysis
1/2/01	156 (18)	Onions beginning tops-down stage
11/2/01	166 (28)	Onions ~90% tops down in N treatment area, ~40% tops down in spatial area
13/2/01	168 (30)	Applied spore suspension to N treatment Beds 6 and 7
14/2/01	169 (31)	4th sampling of trial (spatial area + N treatment bed 1 only)
15/2/01	170 (32)	Spore trapping commenced
16/2/01	171 (33)	Leaf samples from N treatment Bed 7
16/2/01	171 (33)	Bulb measurements taken from N treatment Bed 7
26/2/01	181 (43)	5th sampling of trial - Spatial analysis leaf sample
3/3/01	186 (48)	30 onions collected from each quadrat and dried on gravel pad
5/3/01	188 (50)	Crop lifted
2/4/01	216 (78)	Moved cured onions from gravel pad to shed
25/6/01	300 (162)	112 days from lifting

[A]Days after sowing

[B]Days after application of inoculum

8.2.6 Irrigation

Irrigation was applied to the entire crop using a travelling gun irrigator until 7/12/00. The travelling gun irrigator moved along the irrigator run (planted with grass) at approximately 35 m/hr, applying water within a diameter of 50 m. After 7/12/00, additional irrigation was applied to the high irrigation experiment area with monsoon irrigators run on fixed 100 mm irrigation pipe (Figure 8.2). The monsoon irrigators were placed 9.4 m and 16.6 m apart, measured from the edge of the irrigation buffers, between Beds 6 and 7 of the nitrogen treatment area and between Beds 7 and 8 of the spatial analysis area, for both the high and low irrigation experiment areas. The monsoon irrigators applied water within a diameter of approximately 28 m. A schedule of irrigation events is provided (Table 8.4).

Figure 8.2: Monsoon sprinklers in the nitrogen treatment area (spatial analysis area to the right)

Table 8.4: Irrigation applied to the onion crop in Field trial 2.

Date	Top or all crop[1]	Duration
5/12/01	Traveller	-
7/12/00	Top half	1 hr
13/12/00	Monsoon all	2 hr
15/12/00	Traveller	-
16/12/00	Monsoon all	3 hr
23-25/12/00	Traveller	-
27/12/00	Monsoon all	1 hr
2/1/01	Monsoon all	2 hr
3/1/01	Monsoon all	2 hr
5/1/01	Traveller	-
10/1/01	Top half	2 hr
16/1/01	Top half	2 hr
18/1/01	Monsoon all	3 hr
19/1/01	Top half	5 min
21/1/01	Top half	10 min
22/1/01	Top half	1 hr
23/1/01	Top half	20 min
2/02/01	Monsoon all	3 hr
6/2/01	Top half	2.5 hr
7/2/01	Top half	5 min
10/2/01	Monsoon all	3 hr
20/2/01	Top half	5 min
21/2/01	Top half	10 min
22/2/01	Top half	10 min
24/2/01	Top half	5 min

[1]Indicates what was used to apply irrigation and the area covered. Traveller = the travelling irrigator that irrigated the entire trial; Top half = monsoon irrigators that were used to irrigate the trial in only the high irrigation experiment area; Monsoon all = monsoon irrigators used to irrigate the entire trial (i.e., both high and low irrigation experiments).

8.2.7 Nitrogen treatment and sampling procedure

Nitrogen treatment plots were 1.4 m long with a 1 m buffer (along beds) between treatments (Table 8.5). Of the seven onion beds in the nitrogen treatment area, Beds 1, 4 and 7 received the nitrogen treatments, and the beds between these acted as buffers.

Two nitrogen treatments were tested. A base application of urea (46% N) was applied at a rate of 128 kg/ha to the entire crop on the 5/12/00 using a spreader attached to a tractor. The high nitrogen treatments received an additional 90 kg/ha of urea (46% N), by applying 20.1 g per plot by hand.

Table 8.5: Layout of nitrogen treatments in the nitrogen treatment area. Onion beds are depicted down the page. The area above the irrigation buffer was the low irrigation experiment area, while the area below the irrigation buffer was the high irrigation experiment area (beds were 1 m wide, with 0.6 m alleys between beds).

	Bed 1	Bed 2 – buffer	Bed 3 - buffer	Bed 4	Bed 5 - buffer	Bed 6 - buffer	Bed 7
Low irrigation experiment	N+ R1			N- R2			N+ R1
	N+ R4			N- R1			N+ R2
	N- R5			N+ R5			N- R2
	N- R1			N+ R2			N+ R3
	N- R4			N+ R3			N- R4
	N+ R3			N- R4			N- R5
	N+ R5			N- R5			N+ R5
	N+ R2			N+ R4			N+ R4
	N- R3			N- R3			N- R3
	N- R2			N+ R1			N- R1
Irrigation Buffer (10 m)							
High irrigation experiment	N- R1			N+ R4			N- R2
	N- R3			N- R3			N- R1
	N+ R4			N+ R5			N+R4
	N+ R2			N- R4			N+ R3
	N+ R1			N- R5			N- R4
	N- R4			N+ R3			N- R3
	N- R5			N+ R2			N+ R2
	N- R2			N+ R1			N- R5
	N+ R5			N- R1			N+ R1
	N+ R3			N- R2			N+ R5

N+ = high nitrogen treatment

← N

N- = base (low) nitrogen treatment

R# = replicate number (n=5)

Nitrate sap analysis

Plant samples were collected for nitrate sap analyses at 6.30 am on the 1/2/01. The agricultural consulting company Serve-Ag Pty. Ltd., Devonport, tested the inner leaf samples for sap nitrate. Within the nitrogen treatment area, 3 replicate samples were collected per nitrogen treatment in the high and the low irrigation experiments (Table 8.5), by taking composite samples of 5 leaves per replicate. Non-replicated composite samples of 5 leaves were collected from the infection bed and from Beds 2-10 of the spatial area from within the high and the low irrigation experiment areas (Table 8.6). Results for the nitrate levels were analysed by ANOVA using Genstat®. Statistical tests were not performed on the nitrate samples taken from the spatial analysis area, as no replicates samples were collected (Table 8.8).

Table 8.6: Samples collected for nitrate sap analysis on the 1/2/01.

Number of replicates	Irrigation experiment	Nitrogen or Spatial treatment area
3	High irrigation	High Nitrogen
3	High irrigation	Low Nitrogen
3	Low irrigation	High Nitrogen
3	Low irrigation	Low Nitrogen
1	High irrigation	Infection bed
1	Low irrigation	Infection bed
1	High irrigation	Spatial area (Beds 2-10)
1	Low irrigation	Spatial area (Beds 2-10)

Measurements of onion bulbs

Measurements were taken of bulbs collected from the nitrogen treatments to ascertain differences in bulb size and shape as a result of extra nitrogen application. Bulbs were collected from nitrogen treatment Bed 7, by collecting all bulbs from within a 1 m section of the bed within the treatment plots from both irrigation experiments. Three bulb measurements were taken: bulb diameter, neck diameter and top diameter.

Bulb diameter was measured across the widest part of the bulb. Neck diameter was measured at the point where the vernier clamp sat comfortably on the neck of the bulb. Top diameter was measured 30 mm above the neck of the bulb. All bulbs were measured within the 1-m section in each plot, with five replicate plots per treatment for the bulb and neck diameter measurements, and three replicate plots per treatment for the top diameter measurements. The bulb diameter, bulb neck diameter and the ratio of bulb width to neck width was determined and assessed by ANOVA using Genstat®.

Bulb sample stored for determination of neck rot

A sample of 30 bulbs was collected from each of the quadrats in the nitrogen treatments in Bed 1 on the 3/3/01. These bulbs were cured, stored and assessed for *B. allii* neck rot in a similar manner to the bulb sample taken for the spatial analysis study (Section 8.2.8)

8.2.8 Spatial analysis sampling procedure

Leaf samples were collected at the times indicated in Table 8.3. For samples 1-4 in the spatial area, 20 leaves were collected from 6 randomly-selected quadrats from Beds 1, 2, 4, 6, 10 and 14 of both the high and low irrigation experiments. For the nitrogen treatment area, 20 leaves were collected per quadrat from Beds 1, 4 and 7, unless stated otherwise.

In the spatial analysis area, a leaf sample was taken on the 26/2/01 in a symmetric fractal pattern within quadrats, as demonstrated in Figure 8.3, across the entire 10 x 10 lattice of quadrats in both the high and low irrigation experiments. The sample pattern

within the quadrats was in Rows 2, 5 and 8, and plants were sampled within the rows at 0.1, 0.5, 0.9, 1.0, 1.1, 1.5 and 1.9 m, measured from the start of each quadrat, which faced the irrigation buffer (Figure 8.1) (plants 1, 5, 9, 10, 11, 15 and 19). This pattern was repeated in each of the quadrats (Figure 8.3).

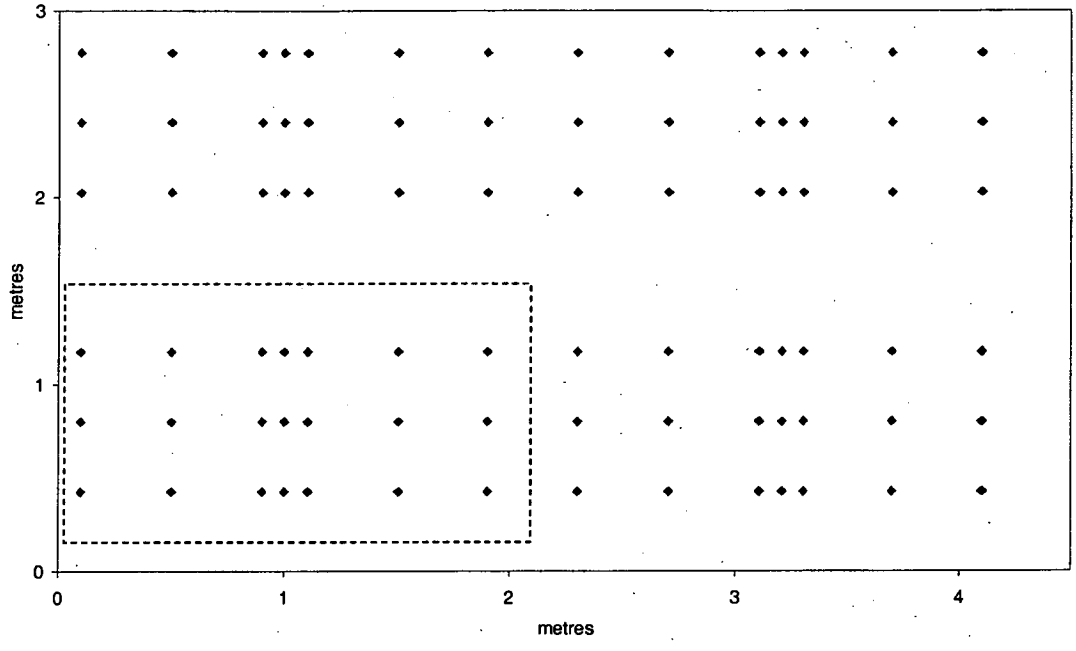

Figure 8.3: Fractal sampling pattern used in Field trial 2. Broken line indicates a single quadrat. Onion beds were orientated along the x-axis. ♦ = sampling location.

All leaf samples were incubated as previously described in Chapter 6 and observed for the presence of *Botrytis* conidiophores.

8.2.9 Bulb storage conditions

Thirty onion bulbs were collected in onion bags on the 3/3/01 from all of the spatial analysis quadrats. The bulb samples were cured on a gravel pad 1 km from the field trial site before being placed into storage on the 2/4/01. A sample of 69 onion bulbs was also collected within the infection bed, 15 m from the edge of the high irrigation experiment, and cured and stored as previously described. Bulbs were stored in

wooden crates in a potato storage shed in which the ambient temperature ranged from 10 – 14°C and RH ranged from 70 to 98% measured using a Watchdog 450 data logger (described previously).

On the 25/6/01 (112 days after lifting) the bulbs were cut in half and assessed for *B. allii* bulb rot symptoms.

8.2.10 Spatial and temporal analysis techniques

8.2.10.1 Disease maps

Disease maps were constructed from samples collected on the 5th sampling period at 43 days after inoculum application. Disease maps were prepared using Microsoft Excel®, by entering XY position coordinates for each infected plant sample.

8.2.10.2 Disease progress curves

Disease progress curves were plotted for the high and low irrigation experiments for both *Botrytis* spp. using samples taken in Beds 2,4,6 and 10. Bed 1 was excluded from the *B. allii* disease progress curves, as it was the inoculum source.

8.2.10.3 Disease gradients

Disease gradients were calculated from the percentage incidence of infected plants determined on the third, fourth and fifth sample times, for both the high and low irrigation experiments. Disease gradients were fitted to inverse power law and

negative exponential law models. For further details of these analysis methods see Chapter 7.

8.2.10.4 Spatial analysis

Ordinary runs analysis (ORA), beta-binomial distribution (BBD), spatial analysis by distance indices (SADIE) and radial correlation analysis were performed as described in Chapter 6. For radial correlation analysis, analysis was not performed on the *B. allii* data set, as the epidemic did not yield a higher incidence of *B. allii* infection than the previous trial (Field trial 1). However, the high incidence of *B. cinerea* in the high irrigation experiment enabled radial correlation analysis to be performed on this data.

8.2.11 Weather data

Air temperature, RH, leaf wetness and rainfall were measured using a data logger (WatchDog model 450), as described previously (Chapter 6, Section 6.2.4). Weather data was collected in both irrigation experiments once the trial was initiated.

8.3 Results

8.3.1 Seed test and transmission of *B. allii* infection in the field

The 'infected' seed lot was tested for *B. allii* infection as per Appendix 12.4. The incidence of seed infection with *B. allii* was 28.9% ± 5.5% standard deviation, determined from 5 replicates. *Botrytis allii* was not detected in seed lot 9-C-175 and testing by Serve-Ag Pty. Ltd. also showed no *B. allii* in that seed line. On the 4/12/00, only one infected plant was detected in the bed sown with infected seed, out of a total

of 740 leaf samples collected from the 22/11/00 to 8/1/01 (Table 8.2). After a second application of a spore suspension of *B. allii* (14/1/01), 41% and 43% of plants sampled from the infection bed were infected with *B. allii* in the high and low irrigation treatments, respectively, as determined by the leaf samples collected on the 15/1/01.

8.3.2 Viability of inoculum

For the spore suspensions applied to the field trial infection bed on the 14/1/01, approximately 50% of the spores formed germ tubes when the suspension was incubated on 1% water agar in Petri plates for 12 hours.

8.3.3 Nitrogen treatment

Plant sap nitrate

Application of additional nitrogen (high nitrogen treatment) resulted in differences in the onion leaf sap nitrate concentration significantly compared to the normal nitrogen treatment in both high and low irrigation experiments (Table 8.7).

Table 8.7: Sap nitrate concentration (ppm) in the high and low irrigation and normal and high nitrogen plots, taken 156 days after sowing (3 replicates).

Measurement	Irrigation experiment	High nitrogen	Normal nitrogen	F probability	LSD
Nitrate (ppm)	High	161.70	124.00	0.02	20.08
Nitrate (ppm)	Low	157.70	127.00	0.01	12.25

No statistics were performed on the level of nitrogen in the spatial analysis area or the infection bed as only a single replicate was collected from these areas (Table 8.8)

Table 8.8: Sap nitrate concentrations (ppm) in the high and low irrigation experiment areas of the spatial analysis study taken 156 days after sowing.

Experiment and area tested	Sap nitrate (ppm)
High irrigation – Spatial area excluding infection bed (Beds 2-10)	110
Low irrigation – Spatial area excluding infection bed (Beds 2-10)	123
High irrigation – Infection bed (Bed 1)	150
Low irrigation – Infection bed (Bed 1)	130

Botrytis allii incidence in nitrogen experiments

The effect of nitrogen on *B. allii* incidence was not determined because *B. allii* infection was not detected in the 4700 leaf samples taken from the nitrogen treatment experiments over a number of sampling times (Table 8.9).

Table 8.9: Incidence of *B. allii* detected in leaf samples collected from the nitrogen treatment areas.

Date of assessment	Nitrogen plots assessed in the high and low irrigation experiments	Number of leaves with *B. allii*/ number of leaves sampled
22/11/00	All nitrogen plots	0/1200
19/12/00	Nitrogen Bed 1- (random sample)	0/50
4/1/01	10 leaves per plot from nitrogen Bed 1	0/200
8/1/01	Nitrogen Bed 1 –random leaf sample	0/50
15/1/01	All nitrogen plots x 20 leaves/plot	0/1200
31/1/01	All nitrogen plots x 20 leaves/plot	0/1200
14/2/01	Nitrogen Bed 1 (20 leaves per plot)	0/400
16/2/01	Nitrogen Bed 7 (20 leaves per plot)	0/400

Neck rot determined after storage period

A low incidence of *B. allii* bulb rot was found in nitrogen treatment Bed 1, but no significant differences (P=0.05) were found between the nitrogen treatments in the high and low irrigation experiments (Table 8.10).

Table 8.10: Number of bulbs that developed *B. allii* bulb rot in storage.

Experiment	Treatment	[1]Number of bulbs with *B. allii* bulb rot (%) (n=150)
High irrigation	High nitrogen	6 (4%)
	Normal nitrogen	6 (4%)
Low irrigation	High nitrogen	12 (8%)
	Normal nitrogen	6 (4%)

[1] 5 replicates of 30 bulbs per treatment

Measurements of onion bulbs

Analysis of bulb measurements on bulb samples collected from bed 7 demonstrated that no significant difference existed between the nitrogen treatments (Table 8.11). Although no effect of nitrogen could be statistically proven, a trend appeared in the data set. All of the high nitrogen treatment measurements were higher than the normal nitrogen measurements, suggesting that the high nitrogen treatment increased the diameters of the onion top, bulb and neck. The bulb diameter:neck diameter ratio was also not statistically different, although in the high nitrogen treatments the bulb:neck ratio was lower, suggesting that the bulbs were bigger proportional to the neck (Table 8.11). The difference between the irrigation experiments could not be statistically tested as they consisted of one replicate. However, it appears as though the high irrigation experiment resulted in larger diameters for the onion top, bulb and neck diameters. Likewise the bulb diameter:neck diameter ratio appears lower in the high

irrigation experiment compared to the low irrigation experiment, suggesting that necks were wider proportional to the bulb diameter in the high irrigation experiment (Table 8.11).

Table 8.11: ANOVA results of nitrogen experiments

Measurement	Irrigation experiment	Nitrogen treatment		F probability	LSD
		High nitrogen	Normal nitrogen		
Top diameter (mm)	High	11.40	8.66	0.30	8.43
Top diameter (mm)	Low	8.09	7.39	0.36	2.60
Bulb diameter (mm)	High	70.70	65.3	0.12	7.50
Bulb diameter (mm)	Low	65.20	62.90	0.28	5.01
Neck diameter (mm)	High	20.73	18.08	0.11	3.62
Neck diameter (mm)	Low	17.64	16.36	0.16	2.03
Bulb:neck ratio	High	3.43	3.62	0.18	0.33
Bulb:neck ratio	Low	3.69	3.85	0.15	0.25

8.3.4 Bulb sample from infection bed

The bulb sample collected from the infection bed (sown with the *B. allii* 'infected' seed) outside of the area that was inoculated with the spore suspensions of *B. allii* did not develop neck rot in any of the 69 bulbs collected.

8.3.5 Disease map

The distribution of *B. allii*-infected plants was concentrated in the infection bed (Bed 1), with some spread of the pathogen into the rest of the sample area (Figure 8.4 and Figure 8.5) at 43 days after the last inoculum application. A higher incidence of *B. allii* leaf infection (although not statistically validated) occurred in the higher irrigation experiment (12%) than in the low irrigation experiment (8%) (inclusive of the infection bed). The incidence of *B. allii* leaf infection (excluding the infection bed) in the high irrigation experiment (4.5%) (Figure 8.5) appeared to be higher than the incidence of *B. allii* leaf infection in the low irrigation experiment (1.9%) (Figure 8.4). The incidence of *B. allii* bulb rot (excluding the infection bed) in the high irrigation experiment (6.7%) appeared to be higher than the incidence of *B. allii* bulb rot in the low irrigation experiment (3.4%).

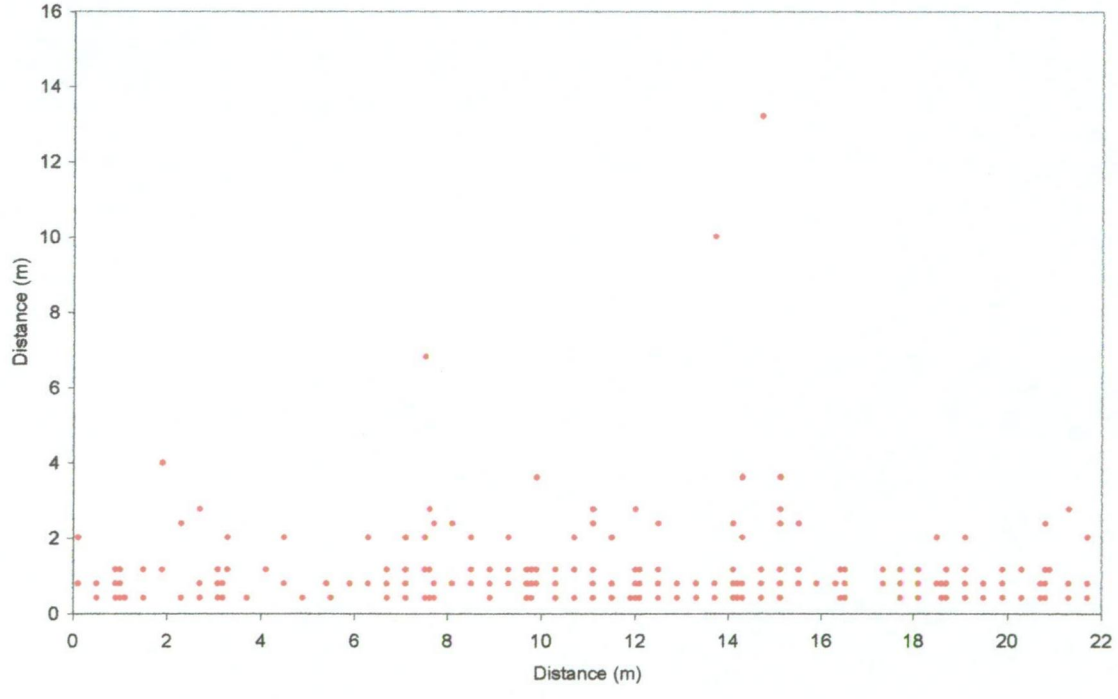

Figure 8.4: Spatial pattern of *B. allii*-infected plants in the low irrigation experiment after 43 days exposure to inoculum (181 days crop age).

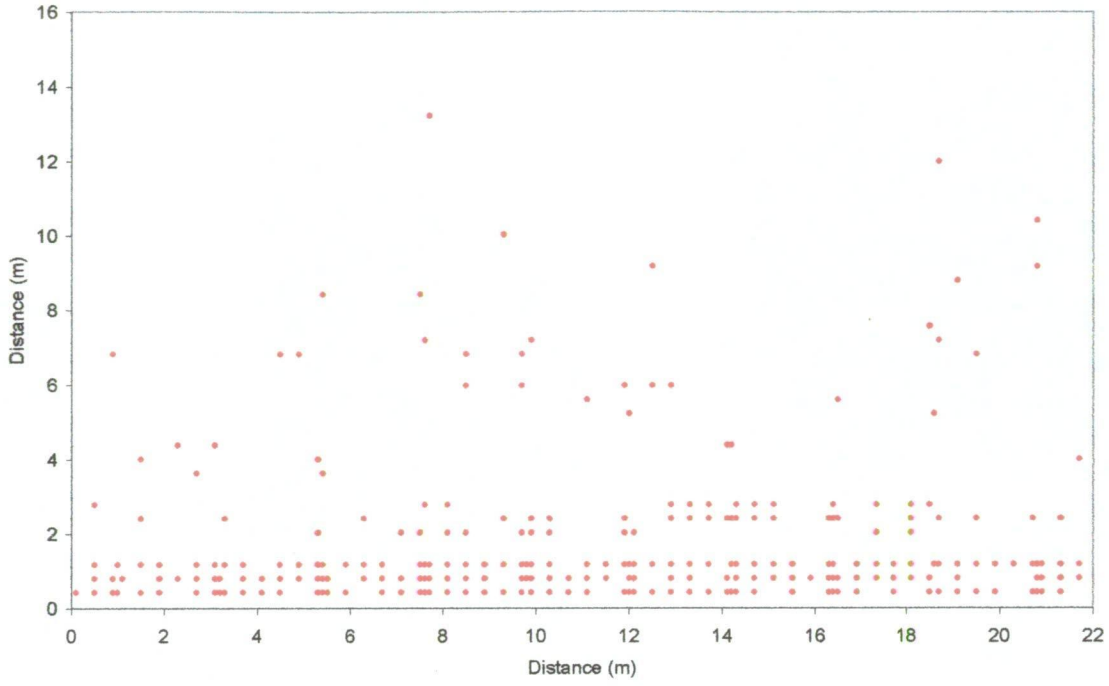

Figure 8.5: Spatial pattern of *B. allii*-infected plants in the high irrigation experiment after 43 days exposure to inoculum (181 days crop age).

The incidence of *B. cinerea* leaf infection in the high irrigation experiment (34.9 %) (Figure 8.7) appeared to be greater than the incidence of *B. cinerea*-infected leaves in the low irrigation experiment, though not statistically proven (18.0%) (Figure 8.6).

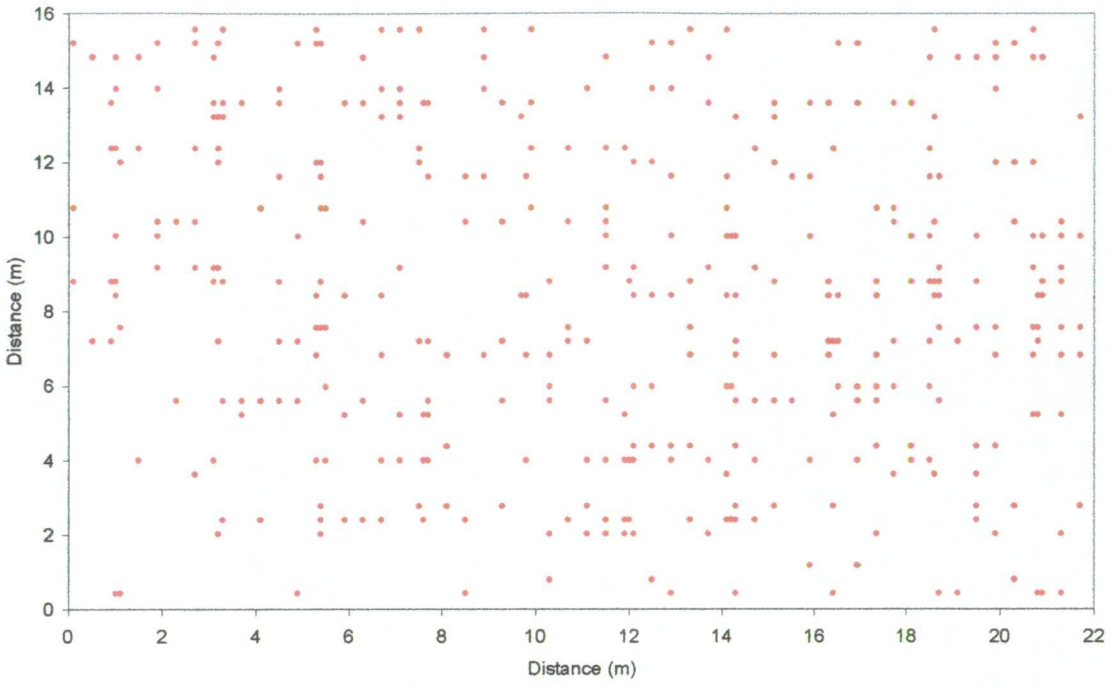

Figure 8.6: Spatial pattern of *B. cinerea* in the low irrigation experiment after 43 days exposure to inoculum (181 days crop age).

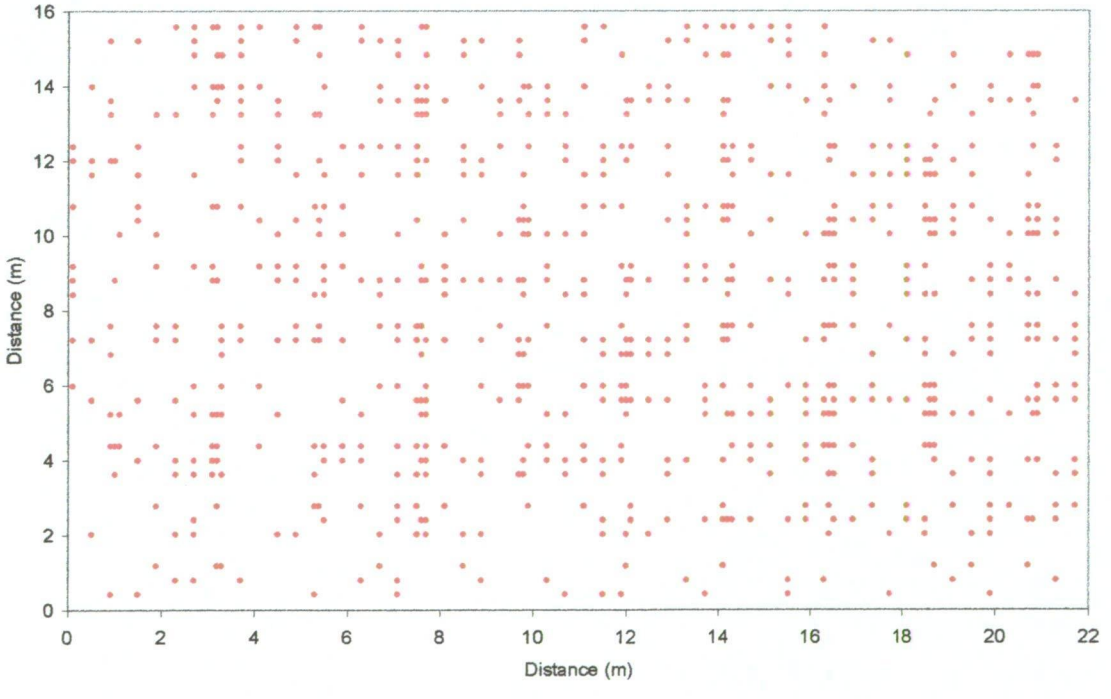

Figure 8.7: Spatial pattern of *B. cinerea* in the high irrigation experiment after 43 days exposure to inoculum (181 days crop age).

8.3.6 Disease progress curves

The disease progress curves plotted in this chapter are non-cumulative, as plants were not sampled consecutively as in Chapter 7. As there were no replicates of the irrigation treatments (experiments), no statistical analysis could be performed on the two treatments. The incidence of *B. allii*-infected plants increased rapidly after application of inoculum at 138 days of crop age (Figure 8.8). The incidence of *B. allii* infection on the low irrigation experiment appeared slightly higher than the high irrigation experiment immediately after the second application of inoculum. However, the incidence of *B. allii* infection in the high irrigation experiment was higher than the low irrigation experiment at the third sampling. At successive assessment dates, incidences of infection were very similar for the two irrigation experiments, with incidence of infection plateauing at the fourth sampling. At the fifth sample period, samples from the low irrigation experiment had an incidence of *B. allii* lower (3%) than that of the high irrigation experiment (7%).

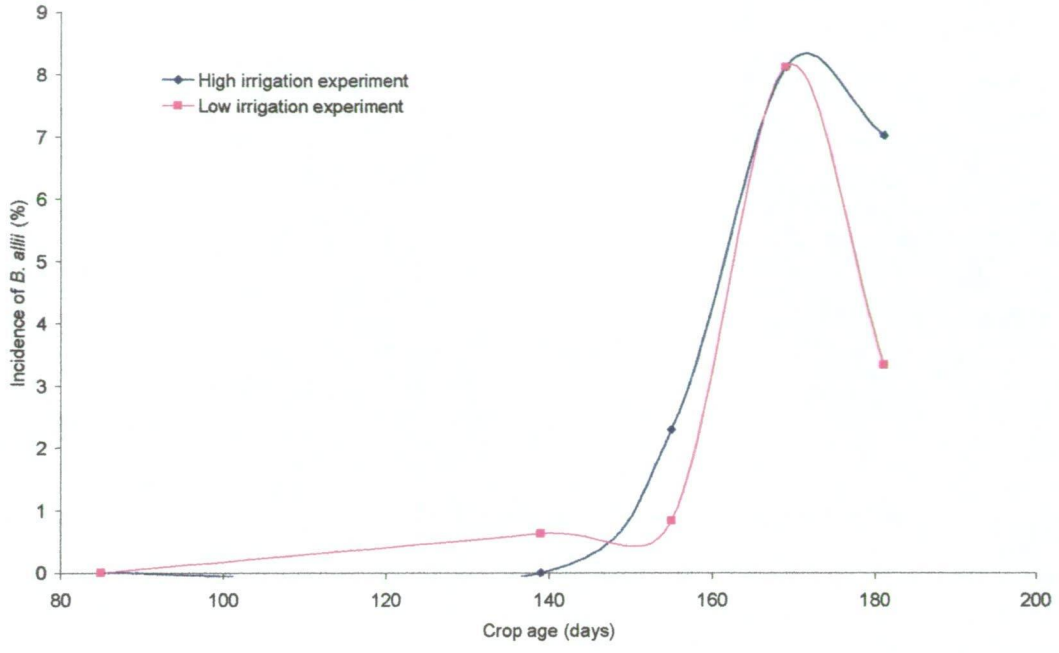

Figure 8.8: Effect of irrigation on the incidence of *B. allii*-infected plants over time, plotted as non cumulative disease progress curves.

The *B. allii* disease progress curves from the high irrigation experiment fit linear, monomolecular and Gompertz models well, with back transformed R^2 values of 0.88, 0.88 and 0.69 respectively (Table 8.12). The *B. allii* disease progress curves from the low irrigation experiment did not fit any of the back transformed models significantly (Table 8.12).

Table 8.12: Fit of disease progress curves of the incidence of *B. allii* in onions from the high and low irrigation experiments.

High irrigation		Linear	Exponential	Monomolecular	Logistic	Gompertz
transformed	F	21.87**	22.3**	21.04**	22.79**	29.67**
	R2	0.88	0.88	0.88	0.88	0.91
	Root MSE	0.016	0.881	0.016	0.89	0.174
	Intercept	0.003	-6.578	0.0	-6.578	-1.879
	rate	0.0019	0.1109	0.0020	0.1129	0.0253
back transformed	F	21.87**	3.02^{NS}	22.39**	3.36^{NS}	6.77*
	R2	0.88	0.50	0.88	0.53	0.69
	Root MSE	0.015	0.056	0.014	0.051	0.029
Low irrigation		Linear	Exponential	Monomolecular	Logistic	Gompertz
transformed	F	2.67^{NS}	6.79*	2.57^{NS}	6.75*	6.41*
	R2	0.47	0.69	0.46	0.69	0.68
	Root MSE	0.028	1.074	0.030	1.095	0.253
	Intercept	0.004	-5.848	0.004	-5.844	-1.754
	rate	0.001	0.075	0.001	0.076	0.017
back transformed	F	2.67^{NS}	0.87^{NS}	2.7^{NS}	0.92^{NS}	1.46^{NS}
	R2	0.47	0.22	0.47	0.23	0.328
	Root MSE	0.019	0.029	0.019	0.029	0.024

NS = Not significant

* = (P = 0.10)

** = Significant (P = 0.05)

*** = Highly significant (P = 0.01)

**** = Very highly significant (P = 0.001)

At the beginning of the experiment, the incidence of *B. cinerea* leaf infection was slightly higher in the low irrigation experiment compared to the high irrigation experiment (Figure 8.9). The incidence of *B. cinerea* was higher in the high irrigation experiment than in the low irrigation experiment only at the last sampling period. The disease progress curves had unusual inflections over the last 3 sampling times (Figure 8.9), with an apparent drop and then an increase in the incidence of leaf infection.

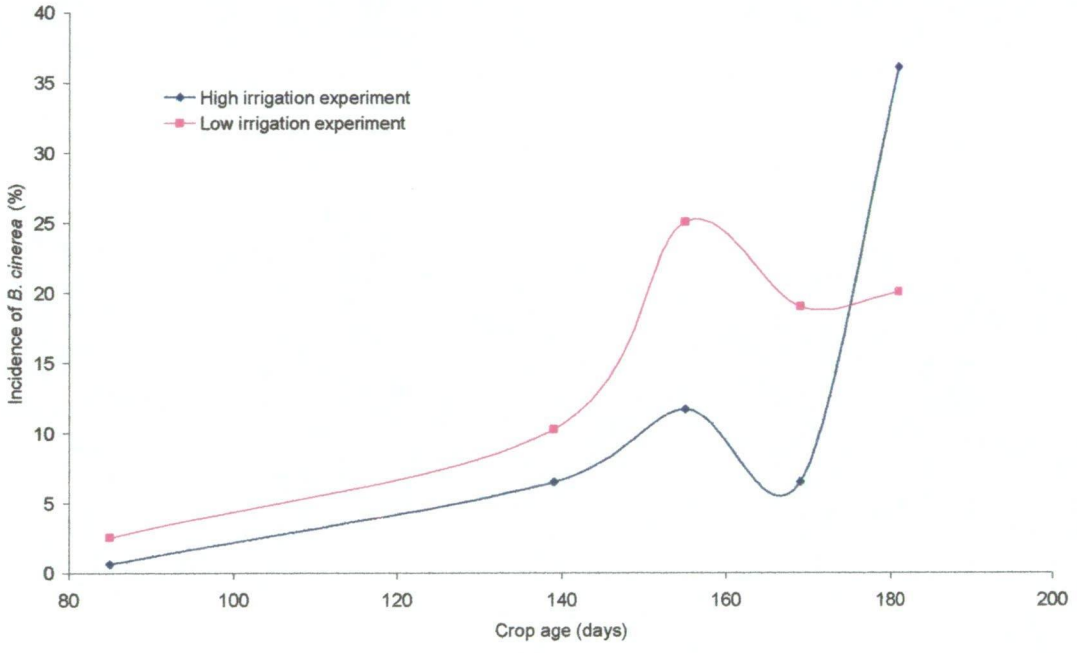

Figure 8.9: Effect of irrigation on the incidence of *B. cinerea*-infected plants over time.

Exponential and logistic models fit the *B. cinerea* disease progress curves in the high irrigation experiment, with R^2 values of 0.75 and 0.72, respectively (Table 8.13). The *B. cinerea* disease progress curves in the low irrigation experiment were fit best by the linear, monomolecular and Gompertz models, with R^2 values of 0.74, 0.75 and 0.70 respectively (Table 8.13), which was opposite to the high irrigation experiment disease progress curve.

Table 8.13: Fit of disease progress curves of *B. cinerea* in onions in high and low irrigation experiments.

High irrigation		Linear	Exponential	Monomolecular	Logistic	Gompertz
Transformed	F	2.81^{NS}	21**	2.44^{NS}	17.39*	8.11*
	R2	0.48	0.88	0.45	0.85	0.73
	Root MSE	0.115	0.581	0.151	0.695	0.342
	Intercept	-0.252	-7.893	-0.315	-8.208	-2.773
	rate	0.003	0.036	0.003	0.039	0.013
backtransformed	F	2.81^{NS}	8.85*	2.52^{NS}	7.62*	5.26^{NS}
	R2	0.48	0.75	0.46	0.72	0.64
	Root MSE	0.080	0.051	0.091	0.054	0.058
Low irrigation		Linear	Exponential	Monomolecular	Logistic	Gompertz
Transformed	F	8.63*	23.37**	7.38*	21.06**	15.37**
	R2	0.74	0.89	0.71	0.88	0.84
	Root MSE	0.052	0.358	0.065	0.416	0.180
	Intercept	-0.145	-5.487	-0.169	-5.657	-2.061
	rate	0.002	0.023	0.002	0.025	0.009
backtransformed	F	8.63*	4.25^{NS}	8.9*	5.04^{NS}	6.87*
	R2	0.74	0.59	0.75	0.63	0.70
	Root MSE	0.045	0.069	0.044	0.063	0.052

NS = Not significant

* = (P = 0.10)

** = Significant (P = 0.05)

*** = Highly significant (P = 0.01)

**** = Very highly significant (P = 0.001)

8.3.7 Disease Gradient

The disease gradients of *B. allii* were steep (Figure 8.10). While there was no indication of the degree of variance around each point within the gradients, the nature of the gradients suggested that more plants were infected with *B. allii* under the high irrigation experiment than the low irrigation experiment. No qualitative differences in shape of the disease gradient curve was noted between the irrigation experiments.

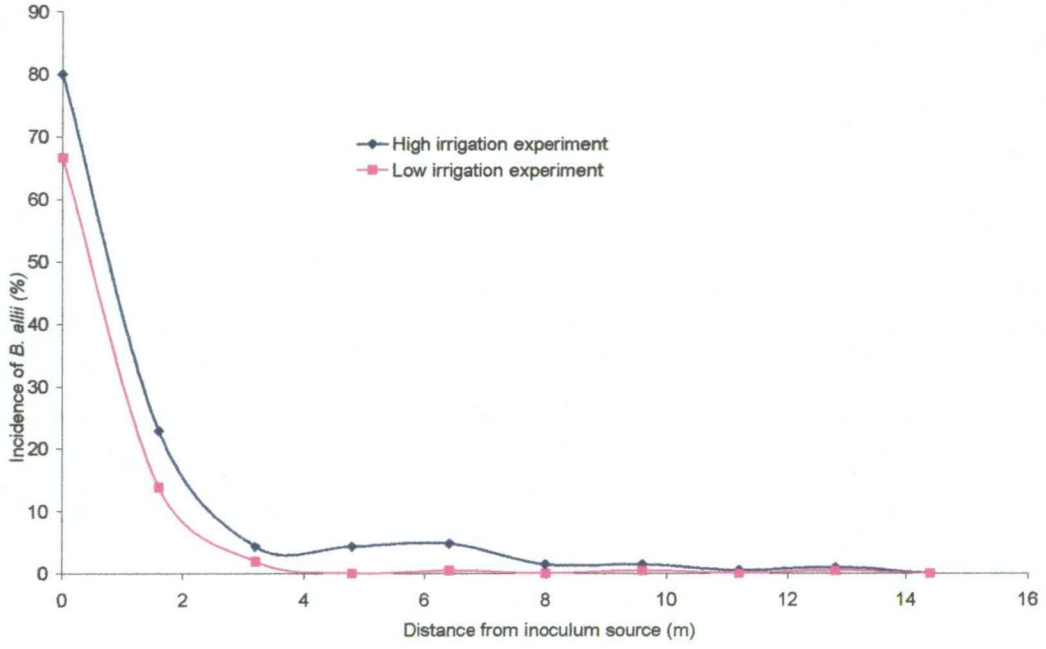

Figure 8.10: Disease gradient of *B. allii*-infected leaves at the 5th sample period, determined on a bed basis (distance from middle of infection bed to centre of beds).

Analysis suggested that the exponential model described the gradients better than the power law model, with higher R^2 values in all the sample times except the third assessment low irrigation experiment where the R^2 values were similar (Table 8.14). The statistical difference between these models was not tested.

Table 8.14: Fit of power law and exponential models for a *B. allii* epiphytotic in onion in high and low irrigation experiments.

	Power law model = A x distance ^{B}			Exponential model = A x exp (-B x distance)		
Time of assessment	R^2	A	B	R^2	A	B
Third (Low irrigation)	0.845	0.015	-0.348	0.844	0.358	1.913
Third (High irrigation)	0.719	0.037	-0.267	0.746	0.434	0.975
Fourth (Low irrigation)	0.728	0.099	-0.182	0.819	0.519	0.493
Fourth (High irrigation)	0.625	0.106	-0.199	0.725	0.662	0.557
Fifth (Low irrigation)	0.882	0.041	-0.302	0.914	0.667	0.992
Fifth (High irrigation)	0.880	0.080	-0.250	0.928	0.800	0.788

8.3.8 Spatial analysis

8.3.8.1 Ordinary runs analysis

The high incidence of *B. cinerea* precluded the use of spatial analysis techniques to describe the spatial pattern of the epiphytotic.

Ordinary runs analysis demonstrated that *B. allii* infected quadrats were distributed non-randomly within and across beds when quadrats were considered contiguous, in both the high and low irrigation experiments (Table 8.15).

Table 8.15: Ordinary runs analysis of *B. allii*-infected onion plants in the high and low irrigation experiments.

Data set	M[1]	N[2]	E(U)[3]	O(U)[4] (within/across beds)	Z-Statistic[5] (within/across beds)
High irrigation	100	44	50.3	28 / 37	-4.44 / -2.61
Low irrigation	100	26	39.5	14 / 17	-6.54 / -5.76

[1] Total number of quadrats

[2] Total number infected quadrats

[3] Expected number of runs

[4] Observed number of runs

[5] Z-statistic = ([observed number of runs + 0.5 – expected number of runs]/standard deviation), with rows treated as contiguous. If Z-statistic is less than –1.64 (P = 0.05), the row of plants has a non-random sequence of infected and healthy quadrats.

8.3.8.2 Beta-binomial distribution analysis

The index of dispersion in the *B. allii* leaf infection and *Botrytis* bulb rot data sets was high (2.0-16.4) relative to the *B. cinerea* data sets (1.3 and 1.8), indicating a stronger aggregation within the former data sets than the latter (Table 8.16). The index of dispersion of the *B. allii* and *Botrytis* bulb rot data sets was much lower when the inoculum bed was removed from the analysis in comparison to the entire data sets. However, the values were still higher than those of the *B. cinerea* data sets (Table 8.16). The LRS indicated that in all but one case, the BBD described the distribution of infected sampled better than the BD. The exception was the *B. cinerea* low irrigation data set which fit both models, but did not have a significant LRS (P = 0.05,

χ^2 3.84) (Table 8.16), and thus was demonstrated not to be a better fit to the BBD than the BD.

Table 8.16: Analysis of *B. allii* and *B. cinerea* epiphytotics by fitting binomial (BD) and beta-binomial distributions (BBD) to the high and low irrigation data sets.

Data set (analysed if > 5% incidence of infection)	Index of dispersion[B]	Goodness of fit Ho: Specified distribution is appropriate		LRS with 1 d.f. P = 0.05, χ^2 3.84[A]
		BBD (non-random)	BD (random)	
B. allii (High irrigation)	11.9	0.000	0.000	645.60
B. allii (High irrigation without source)	4.0	0.055	0.000	90.86
B. allii (Low irrigation)	11.9	0.464	0.000	575.34
B. allii (Low irrigation without source of inoculum)	3.0	0.000	0.000	48.85
B. cinerea (High irrigation)	1.8	0.077	0.003	22.80
B. cinerea (Low irrigation)	1.3	0.508	0.460	2.98
Botrytis bulb rot (High irrigation)	16.4	0.000	0.000	998.93
Botrytis bulb rot (High irrigation without source of inoculum)	4.5	0.223	0.000	140.26
Botrytis bulb rot (Low irrigation)	16.8	0.000	0.000	839.29
Botrytis bulb rot (Low irrigation without source of inoculum)	2.0	0.434	0.025	19.67

[A] LRS in all cases demonstrated that BBD was a better fit than the BD, except for the *B. cinerea* (Low irrigation) data set.

[B] An index of dispersion > 1 indicates aggregation of infected plants.

8.3.8.3 Spatial analysis by distance indices (SADIE)

Spatial analysis using SADIE demonstrated the pattern of *B. allii* and *B. cinerea* infection to be a non-random at all assessment times (Table 8.17).

Table 8.17: Spatial analysis of *B. allii* and *B. cinerea* infection of onion plants by SADIE in the high and low irrigation experiments.

Data set	I_a [1]	P_a [2]
B. allii (High irrigation)	2.378	<0.0002
B. allii (High irrigation – inoculum source removed)	1.919	<0.0002
B. allii (Low irrigation)	2.200	<0.0002
B. allii (Low irrigation – inoculum source removed)	1.748	0.0017
Botrytis bulb rot (High irrigation)	2.339	<0.0002
Botrytis bulb rot (Low irrigation)	2.160	<0.0002
B. cinerea (High irrigation)	1.735	0.0012
B. cinerea (Low irrigation)	1.527	0.0114

[1] Index of aggregation.

[2] Probability that the observed data was no more aggregated than expected from a random permutation of the counts of infected plants.

Table 8.18 provides the legend for the following plots derived from SADIE outputs of clustering indices of the spatial distribution and spatial association between populations of infected onion plants. Confidence of clusters[1] is indicated in red and confidence of gaps is indicated in blue. The 90% and 95% confidence intervals are shown.

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[1] Clusters are defined as areas of significantly more outflows (red) of infected individuals required to achieve regularity, whereas gaps are areas with significantly more inflows (blue) of infected individuals required to achieve regularity. Values outside of the significance ranges, are demonstrated in white.

Table 8.18: Key for levels of significance of clustering of infected plants determined by SADIE.

	95% confidence of outflows
	90% confidence of outflows
	No significant flows
	90% confidence of inflows
	95% confidence of inflows

A positive aggregation of *B. allii* infection extended across the length of the infection bed (21 m) and up to 3 m and 5 m into the trial area in the high and low irrigation experiments, respectively (Figure 8.11 and Figure 8.13, respectively).

Analysis of the data after removal of the source 'bed' of infection indicated significant positive aggregation of *B. allii*-infected plants to a greater distance (9 m) than when including the infection bed in the analyses. Approximately five distinct areas of outflow were seen in the high irrigation experiment (Figure 8.12). In the low irrigation experiment, fewer and smaller areas of outflow were detected (Figure 8.14).

The most northern and the most eastern edge of the trial areas are demonstrated in the following figures.

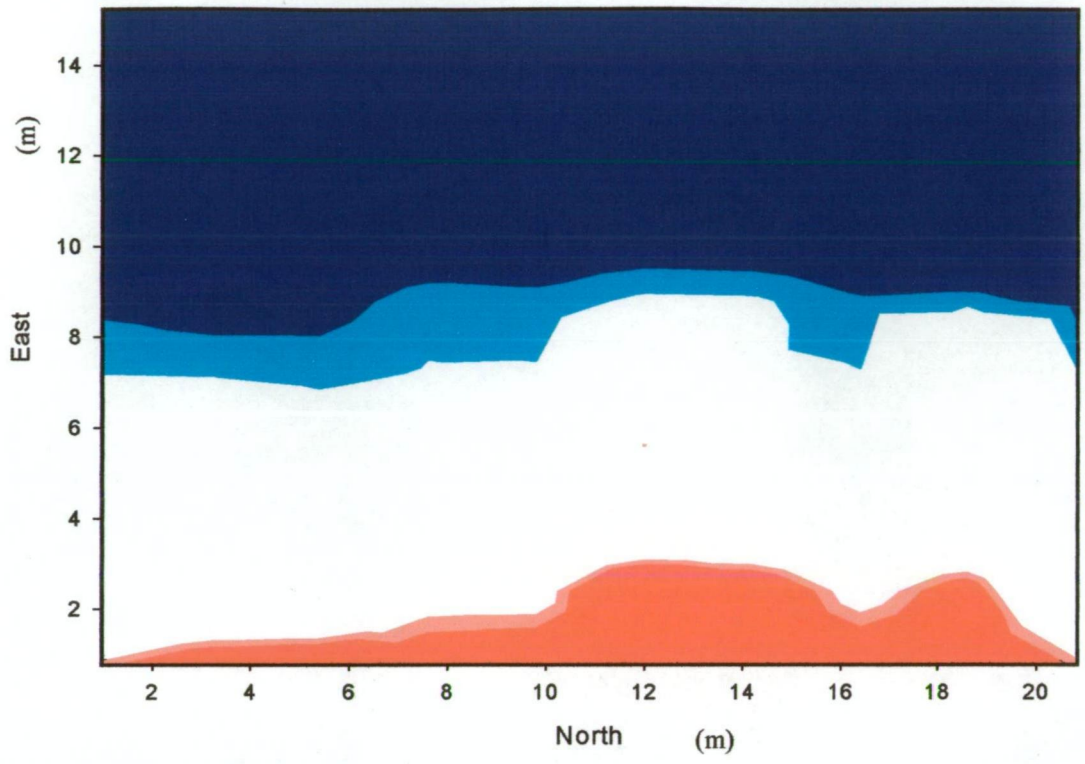

Figure 8.11: Plot of clustering indices of *B. allii* infection in the high irrigation experiment.

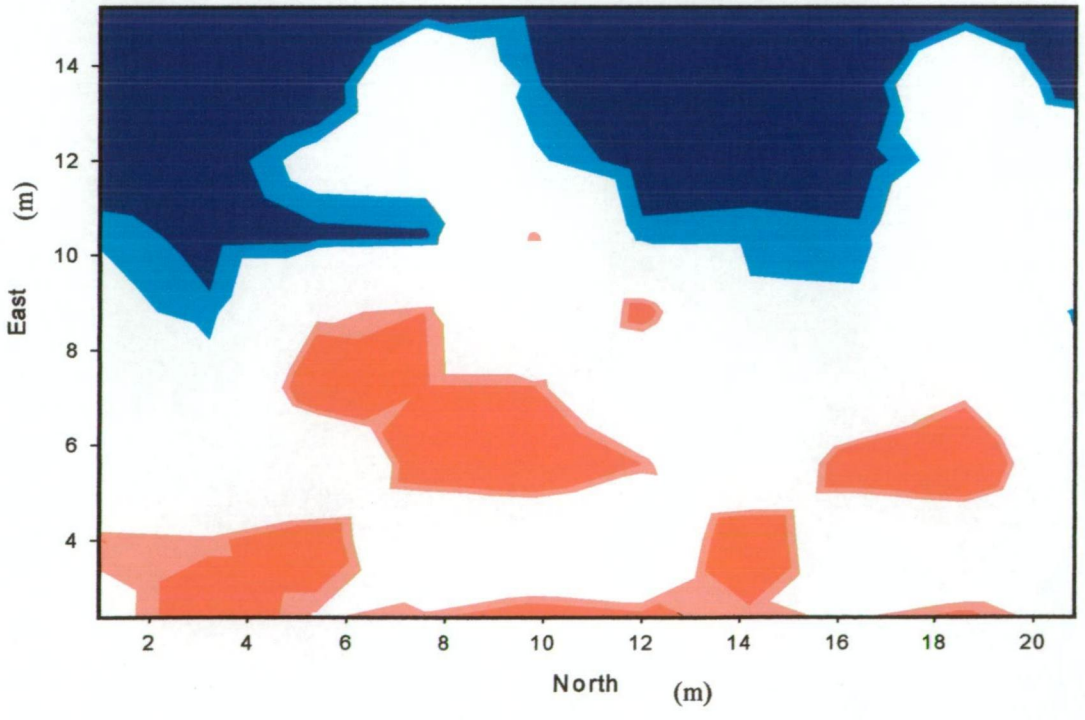

Figure 8.12: Plot of clustering indices of *B. allii* infection in the high irrigation experiment without the infection source in the analysis.

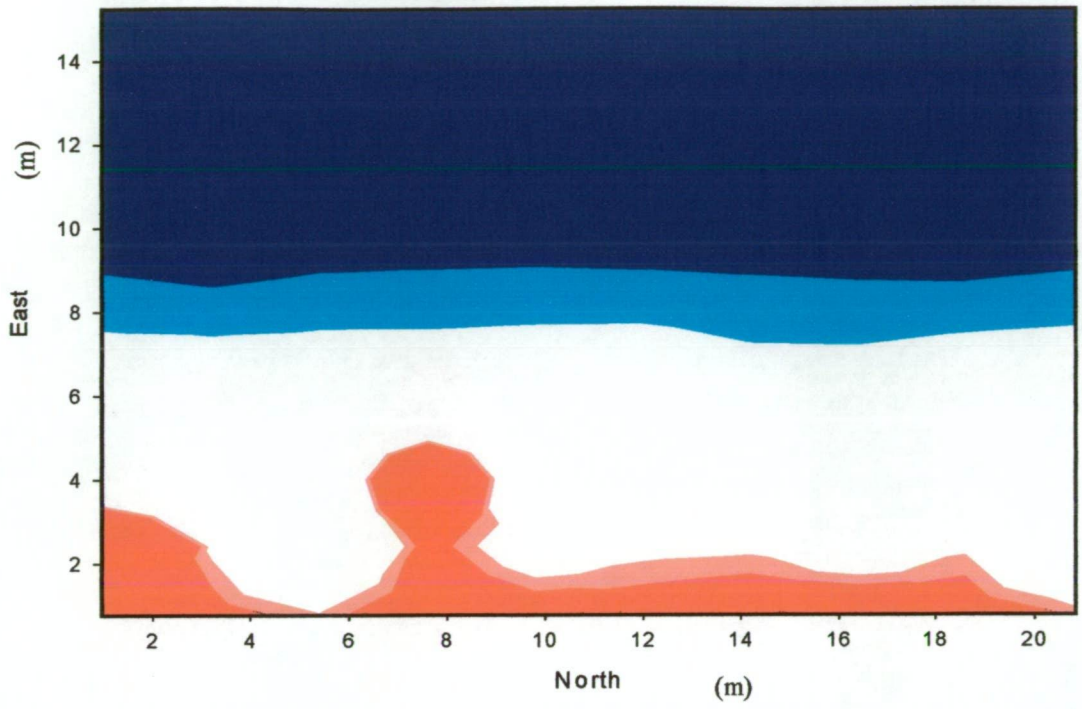

Figure 8.13: Plot of clustering indices of *B. allii* infection in the low irrigation experiment.

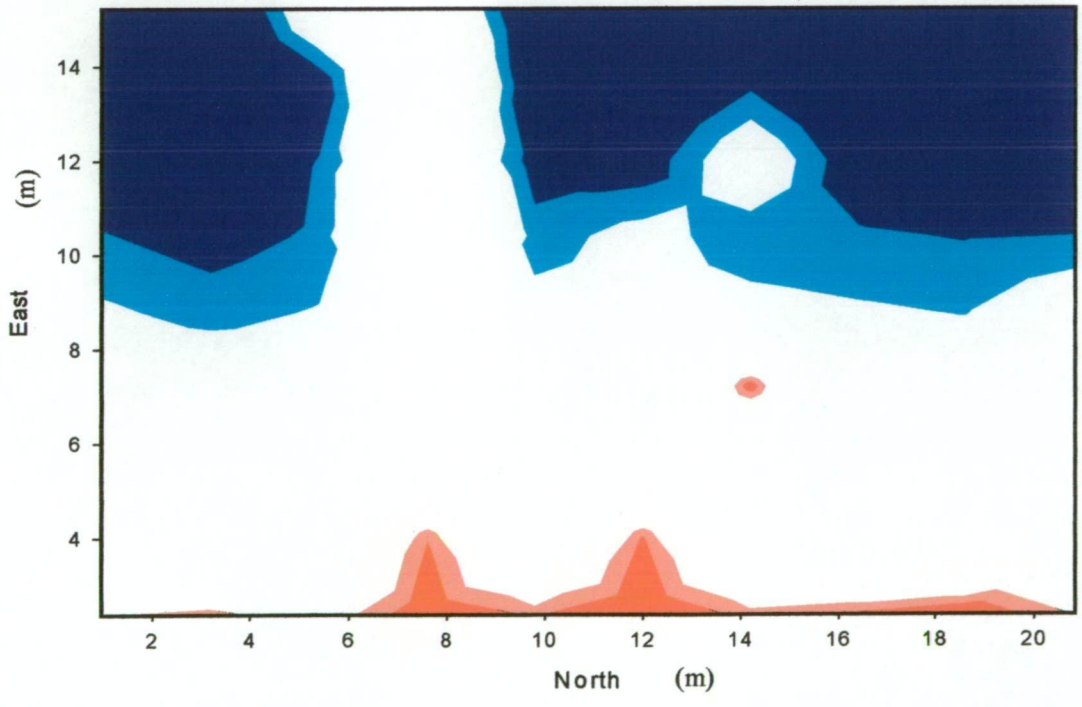

Figure 8.14: Plot of clustering indices of *B. allii* infection in the low irrigation experiment without the infection source in the analysis.

SADIE demonstrated similar cluster plot analyses of the *B. allii* leaf infection data sets as the *Botrytis* bulb rot data sets in the high and low irrigation experiments (Figure 8.15 and Figure 8.16, respectively). The *Botrytis* bulb rot in the high irrigation experiment extended out to 6 m from the northern border, and significant aggregation extended the entire length of the northern border (Figure 8.15). The *Botrytis* bulb rot in the low irrigation experiment only extended slightly into the experiment area, but did extend the length of the northern border (Figure 8.16).

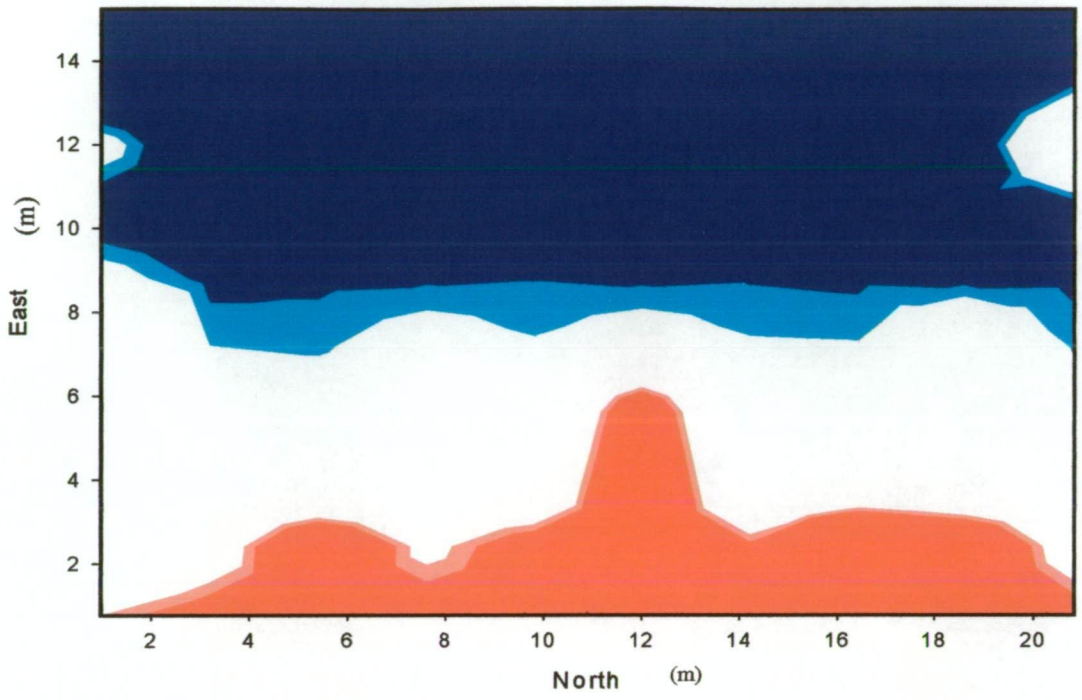

Figure 8.15: Plot of clustering indices of *Botrytis* bulb rot in the high irrigation experiment.

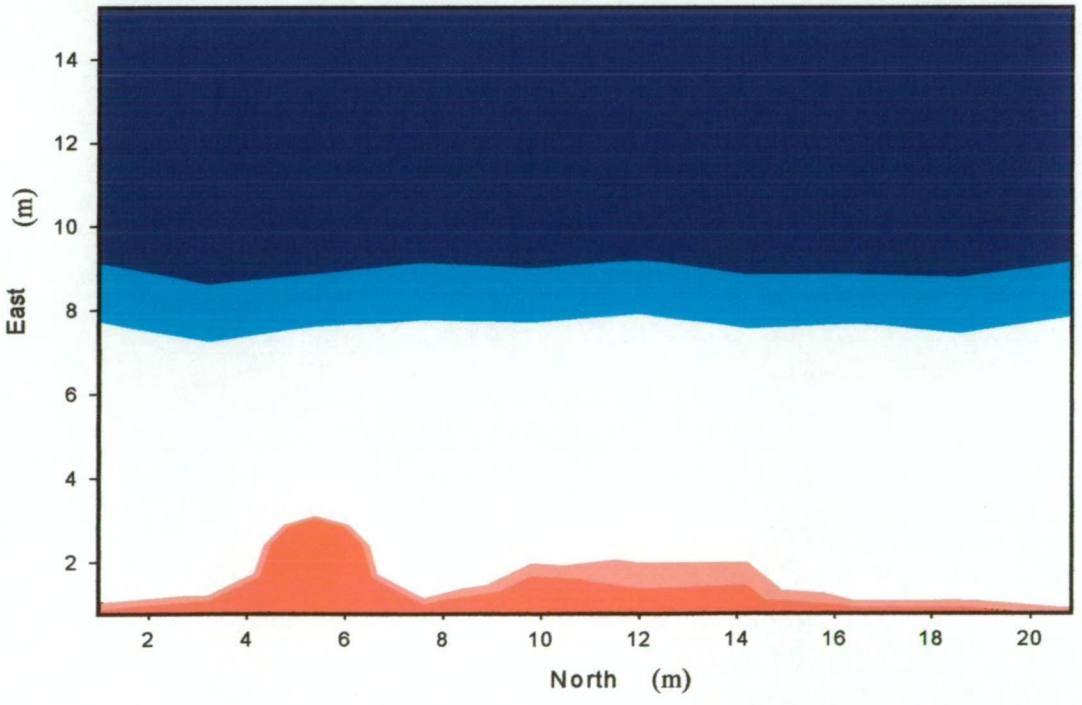

Figure 8.16: Plot of clustering indices of *Botrytis* bulb rot in the low irrigation experiment.

SADIE cluster analysis of the high and low irrigation *B. cinerea* data sets demonstrated clusters of positive association within the plot area (Figure 8.17, Figure 8.18). *Botrytis cinerea* infections did not appear to originate from the *B. allii* inoculum source.

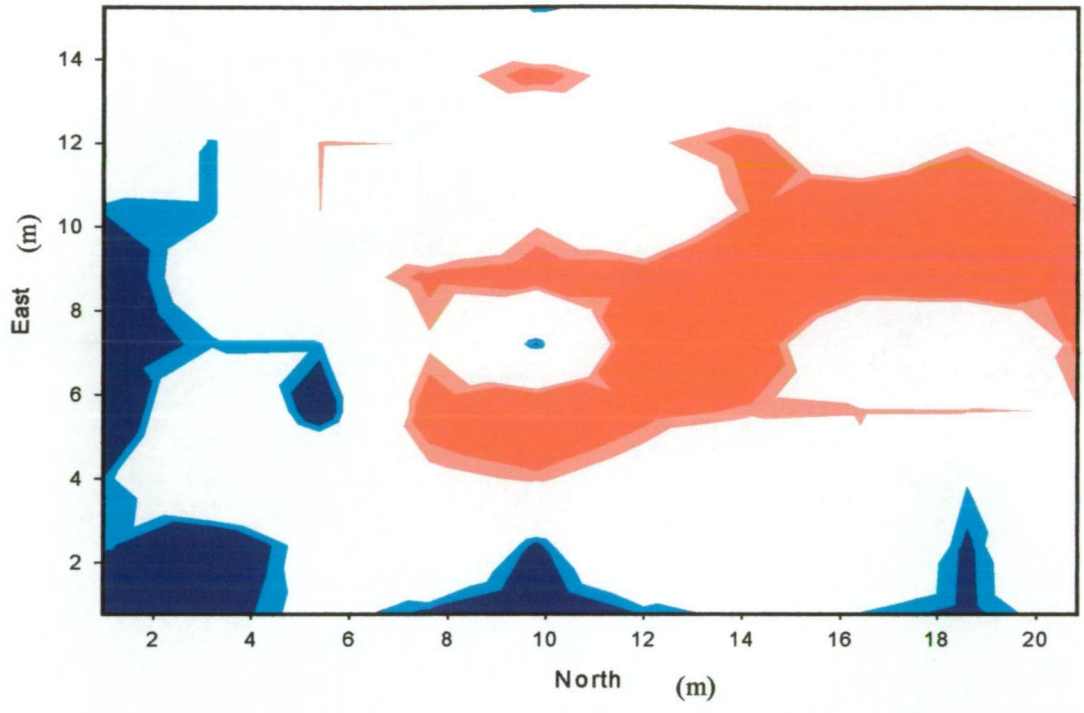

Figure 8.17: Plot of clustering indices of *B. cinerea* infection in the high irrigation experiment.

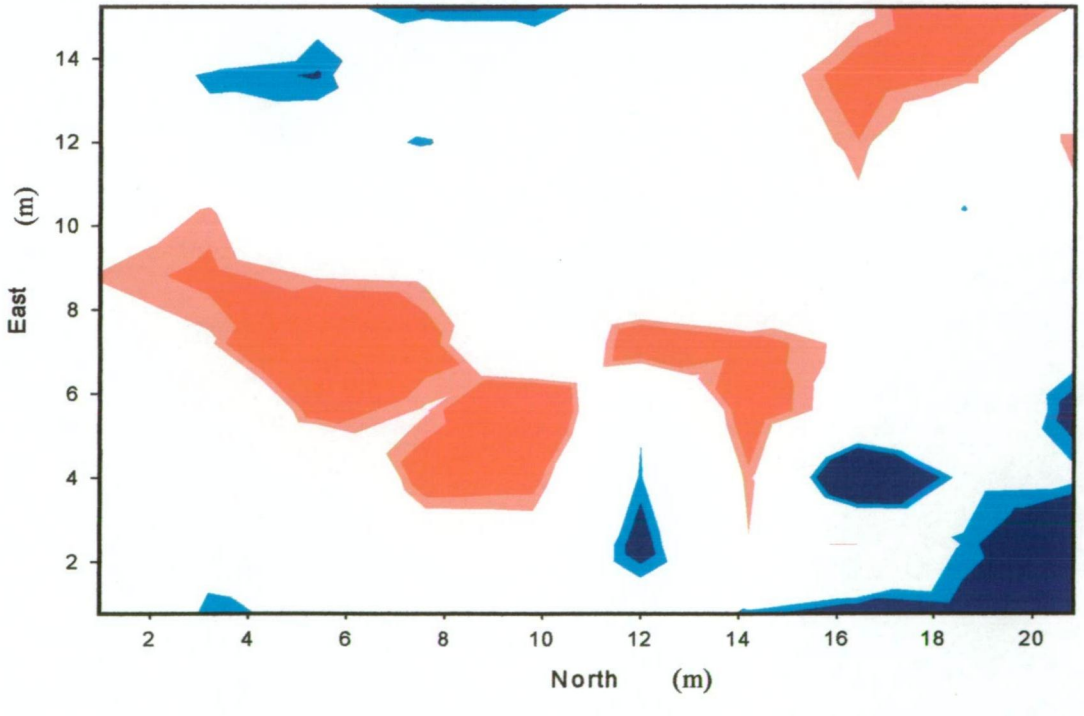

Figure 8.18: Plot of clustering indices of *B. cinerea* infection in the low irrigation experiment.

Spatial association between data sets

Analysis of overall association of infected plants between the data sets (Table 8.19) demonstrated that there was no association between the *B. allii* and *B. cinerea* data sets in the low irrigation experiment (Figure 8.20). However, in the high irrigation experiment there was significant disassociation between *B. allii* and *B. cinerea* in the leaves of the spatial sample taken 43 days after exposure to *B. allii* inoculum (Figure 8.19). Significant association was found between *B. allii* in the leaves and *Botrytis* bulb rot in both irrigation experiments (Figure 8.21 and Figure 8.22).

Table 8.19: Spatial association analysis of *B. allii, B. cinerea* and *Botrytis* bulb rot data sets in the high and low irrigation experiments.

Data set	Overall measure of association (χ)	Probability[A]
High irrigation, *B. allii* and *B. cinerea*	-0.2658 (95% = -0.1953)	0.9967
Low irrigation, *B. allii* and *B. cinerea*	-0.1462 (95% = -0.2037)	0.9182
High irrigation, *B. allii* and *Botrytis* bulb rot	0.8896 (95% = 0.2035)	< .0001
Low irrigation, *B. allii* and *Botrytis* bulb rot	0.8737 (95% = 0.2035)	< .0001

[A]For a (recommended) two-tail test, the probability level should be $P < 0.025$ for a significant association, or $P > 0.975$ for significant dissociation ($\alpha = 0.05$).

Areas of disassociation between *B. allii* and *B. cinerea* in the high irrigation experiment were along the edge of the infection bed (Figure 8.19). A small area of positive association was seen 12 m north and 6 m east within the trial area (Figure 8.19).

Areas of disassociation and association were present between the *B. allii* and *B. cinerea* data sets in the low irrigation experiment. As in the high irrigation experiment, some disassociation was observed alongside the infection bed (Figure 8.20).

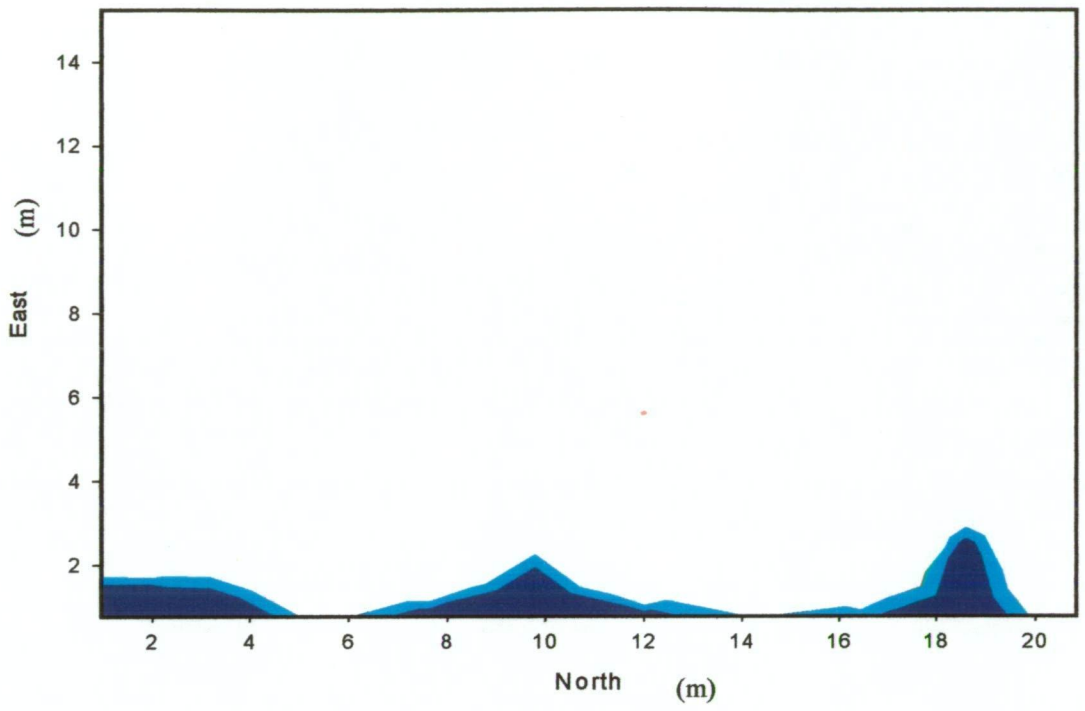

Figure 8.19: Spatial association between *B. allii* and *B. cinerea* in the high irrigation experiment.

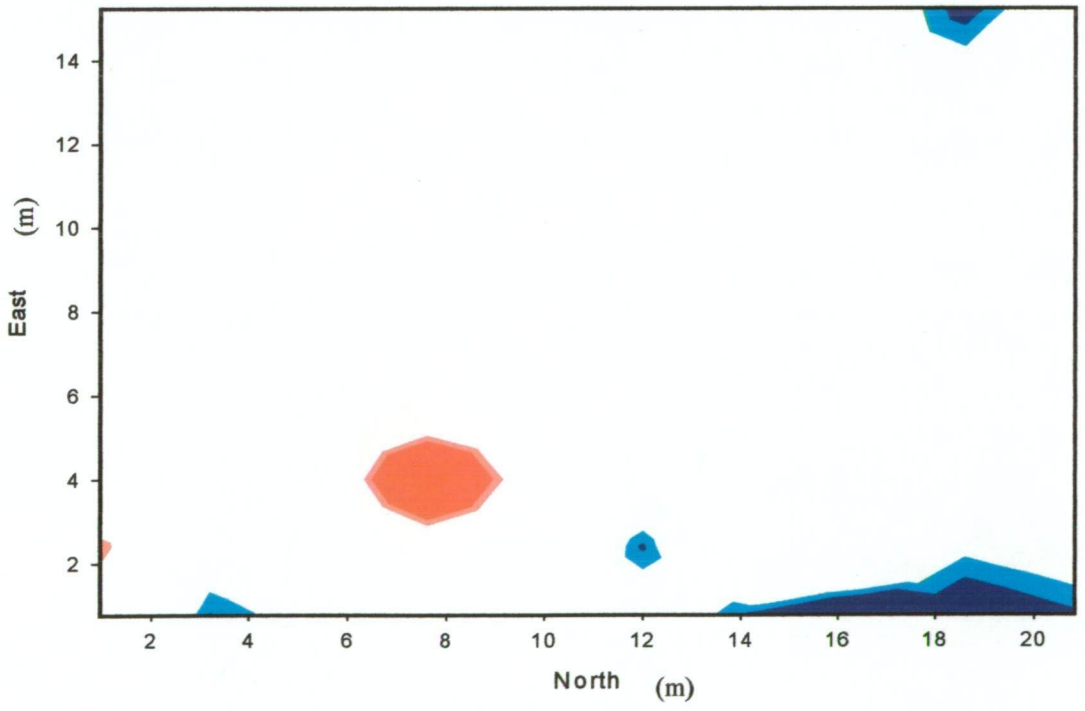

Figure 8.20: Spatial association between *B. allii* and *B. cinerea* in the low irrigation experiment.

No local areas of disassociation were seen in the plots between *B. allii* leaf infection and *Botrytis* bulb rot (Figure 8.21, Figure 8.22). Association between the two variables was observed along the infection bed in both irrigation experiments.

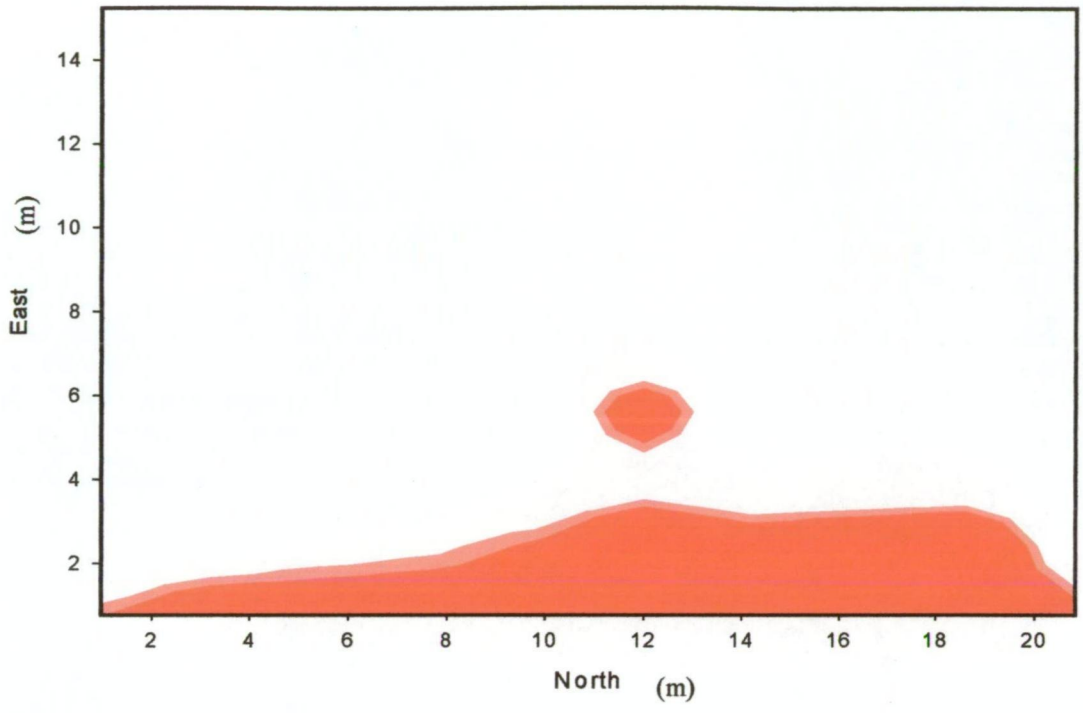

Figure 8.21: *Botrytis allii* and *Botrytis* bulb rot association in the high irrigation experiment.

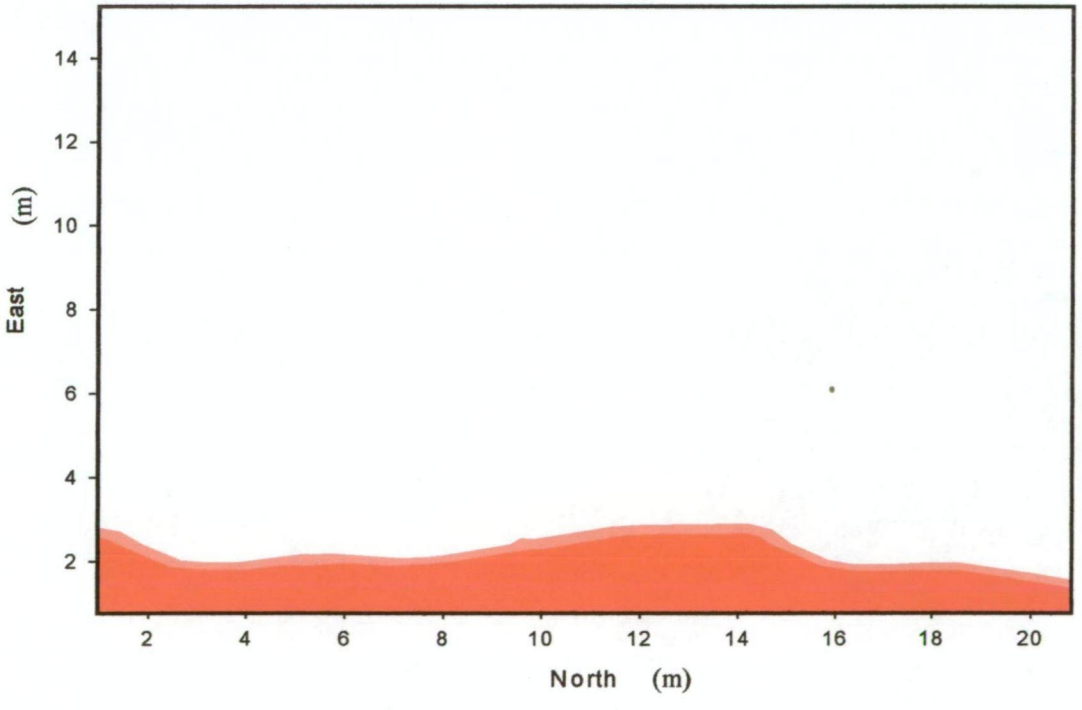

Figure 8.22: Association of *B. allii* and *Botrytis* bulb rot in the low irrigation experiment.

8.3.8.4 Radial correlation analysis of the spatial pattern of *B. cinerea*-infected plants

Radial correlation analysis demonstrated that spread of *B. cinerea* was not symmetrical (Figure 8.23). Infected plant pairs were significantly aggregated ($P < 0.05$) across rows to a distance of < 1 m, and significantly random ($P < 0.05$) at distances > 7 m. Significant aggregation of infected plant pairs occurred within rows up to the 10 m distance class, and a significant random distribution of infected plant pairs was noted at distances up to 17 m within rows. Infected plant pairs in all directions were significantly ($P < 0.05$) aggregated up to 6 m and significantly random at distances > 10 m (Figure 8.23 and Figure 8.24).

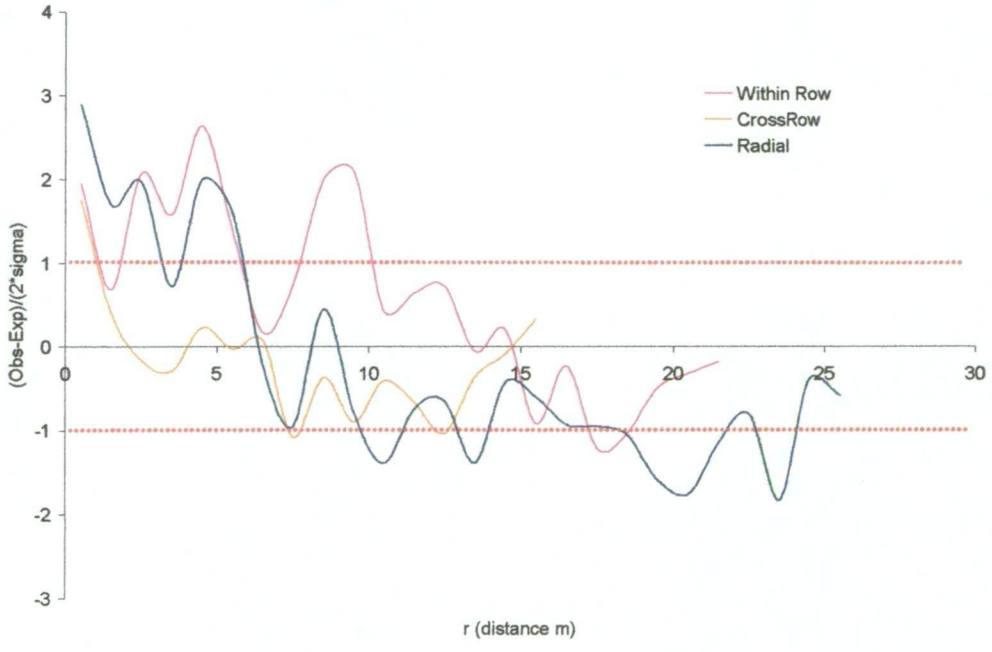

Figure 8.23: Normalised density of *B. cinerea*-infected individuals as a function of radial distance from an infected plant, at 181 days after sowing in the high irrigation experiment.

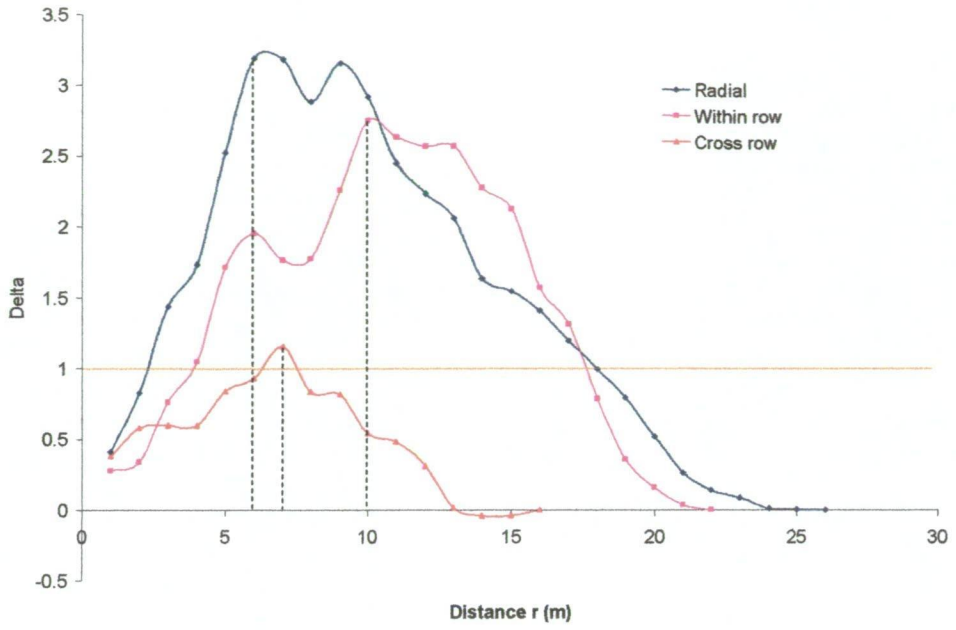

Figure 8.24: Kolmogorov-Smirnov statistic plot of the delta value between the observed and the expected (2DCORR) probability of *B. cinerea*-infected plant pairs against plant pair distances, assessed 181 days after sowing in the high irrigation experiment. The horizontal line at a delta value of 1, indicates deviation from random behaviour at $\alpha = 0.05$.

8.4 Discussion

8.4.1 Seed-to-seeding transmission of *B. allii*

In the field trial, virtually no *B. allii* was transmitted from the *B. allii*-'infected' seed to the onion plants. This was demonstrated by the leaf samples taken before application of spore suspensions of *B. allii* to the plots. The bulb sample collected from the infection bed outside of the area inoculated with the spore suspensions also failed to yield *B. allii*-infected bulbs. Conversely, seed of the same seed lot sown in pots in a greenhouse yielded a high percentage of leaf infection (Chapter 9). This demonstrated that during germination of the onion seed, infection may not be passed from the seed to the seedling under certain environmental conditions. The 'infected'

seed lot was capable of producing a high incidence of *B. allii* seedling infection, as demonstrated in a glasshouse study (Chapter 9).

During germination of onion seed, the seed coat typically adheres to the cotyledon tips. Maude and Presly (1977ab) reported that in the majority of cases mycelium of *B. allii* was found growing from the seedcoat into the living tissue of the tips of the cotyledons. Stewart and Franicevic (1994) also demonstrated that *B. allii* was able to pass from the seed to the resultant seedling, through the same process. In this trial, the environmental conditions during germination appear to have influenced (inhibited) the transmission of *B. allii* from the seed to the seedling. Dry weather conditions during germination of the seed may have prevented germination and growth of the mycelium of *B. allii* from the seedcoat to the seedling.

Maude and Presly (1977a) and Stewart and Franicevic (1994) reported that occasionally the incidence of *B. allii* detected in seedlings was lower than that found in the seeds. No reason was suggested for this phenomenon in either study.

Tylkowska and Dorna (2001) found that incidences of *B. allii* in the seed of < 5% did not guarantee an incidence of infected onion bulbs below the economic threshold if the weather was conducive for disease during growth of the crop. In years less favourable for disease development, they found that an incidence of seed infection > 10% was necessary to cause deterioration in seed quality and plant characters such as germination, 1000 seed weight, emergence, seedling and bulb infection by *B. allii* (Tylkowska and Dorna, 2001). In dry climates, careful irrigation during seed

germination and seedling emergence might provide a management tool to minimise disease incidence and severity.

The critical weather factors that are required for seed-to-seedling transmission of *B. allii* infection are not understood and further studies will need to be performed, perhaps utilising plant growth cabinets.

8.4.2 Field trial disease maps

The spatial pattern of *B. allii*-infected plants suggested short-range spread from the inoculum source (Bed 1). Conversely, the initial spatial pattern of *B. cinerea*-infected plants was random, which is characteristic of a pathogen entering the crop aerially from a distant source of inoculum (Gregory, 1968).

8.4.3 Field trial disease progress curve

Disease progress curves of *B. allii* in this study demonstrated a decline in the number of leaves that were infected late between 169 days from sowing, (31 days after application of inoculum) and lifting of the crop at 188 days after sowing. This may have been due to competition between *B. allii* and saprophytes as the leaves began to senesce, as discussed previously (and in Chapter 9, Section 9.4.3). The rapid increase in both *Botrytis* spp. also appears to be correlated to the tops down stage (where the onion leaf canopy collapses with maturity) as found in Field trial 1 (Chapter 7). This phenomenon requires further investigation, and perhaps control strategies such as chemical or biological control might in the future be timed with this maturity process. Maude *et al.* (1985) also found the peak in the incidence of infected plants in onion

crops occurred towards the end of June, which was towards the end of the onion growing season in the UK. The disease progress curve of *B. cinerea* decreased at the 4th spatial sample (169 days after sowing) but increased again at the 5th spatial sample (181 days after sowing).

The disease progress curves of *B. allii* and *B. cinerea* differed in shape. This was also noted in Field trial 1 (Chapter 7). Perhaps the nature of the pathogenicity of the two species differs, resulting in differences in the disease progress curves, as discussed in Chapter 7. The environmental conditions favouring spore production and plant infection and latent periods etc. by the two species may also differ, resulting in the differences found in this study. Furthermore, the amount of inoculum of each species may have differed resulting in different rates of disease progress for each species.

Given the exponential nature of *B. allii* epiphytotics, it is important to establish onion crops using 'clean' seed. Onion seed is currently tested in Tasmania by plating seeds onto agar in Petri plates, followed by an incubation period to encourage *B. allii* growth onto the agar if present in the seed. Currently, 550 seeds are usually tested at a time per seed lot by the Department of Primary Industries, Water and Environment, Tasmania. If a negative result occurs, this indicates 99% confidence that there is no more than 1% incidence of *B. allii* in the seed (Johnston and Kotz, 1969). Perhaps this level of testing is not adequate given the steep disease progress curves found in this field trial and in Field trial 1 (Chapter 7). To achieve 99% confidence that there is no more than 0.1% *B. allii* in a seed lot, 5000 seeds would need to be tested (Johnston and Kotz, 1969). With the current seed testing procedure this would be very time-consuming. The development of either an ELISA or DNA-based detection method for

B. allii in seed may allow the routine testing of adequately-sized seed samples for greater confidence levels, without the long incubation period needed for assays currently used.

8.4.4 Field trial disease gradient

As in Field trial 1, the exponential model described the disease gradient for *B. allii* better than the power law model. This suggested that the pathogen was either splash dispersed, or some conidia may have been deposited on plants close to the inoculum source while others escaped from the plant canopy by means of dry wind dispersal. The latter mechanism may also result in very steep disease gradients, similar to those caused by splash dispersal (Ferrandino, 1993).

Infection of plants in the infection bed late in the growth of the crop (as a result of spraying spore suspensions of *B. allii* onto the bed) may also have contributed to the steep disease gradient. As there were only 43 days from inoculum application to the final sampling, the disease gradient for the epidemic in Field trial 2 did not progress as far as that of the epidemic in Field trial 1. If disease had been initiated from the original 'infected' seed planted, perhaps the gradient would have been flatter as a result of more spread of infection over time.

8.4.5 Field trial spatial analysis; ORA, BBD, SADIE and Radial Correlation

Spatial analysis techniques demonstrated that the distribution of *B. allii* (43 days after application of inoculum) and *B. cinerea* (181 days after sowing) were not random, and statistically significant aggregations were demonstrated. The spatial analyses for Field

trials 1 and 2 provided similar conclusions. Although the sources of inoculum differed between field trials, the spatial pattern and spread over time of *B. allii*- and *B. cinerea*-infected plants were similar. This is expected given that the species are of the same genus, with many similar characteristics (e.g., conidial size). The differences in sources of inoculum of the two pathogens most likely was associated with some of the differences detected between the species in this study. However, if an onion plot could be established free of *B. cinerea* initially, and the fungus subsequently introduced in a similar fashion to *B. allii* in this study, perhaps similar disease gradients would be seen.

8.4.6 Cultural practices and environmental conditions

The effect of eleven extra irrigations after initiation of an epiphytotic in Field trial 2 was demonstrated to increase (although not statistically validated) the incidence of *B. allii* leaf infection in the final samples, from 8% to 12%. Hassan (1996) reported that onions that received a greater number of irrigation treatments were more susceptible to all the storage pathogens evaluated in that study (*B. allii, Pseudomonas marginalis* and *Erwinia carotovora*) than onions that received fewer irrigation treatments. In the field trial in this study the incidence of infection appeared to be greater in the high irrigation experiment compared to the low irrigation experiment, probably as a result of increasing the infection periods under the former experiment. In addition, the higher irrigation experiment might have made the plants more susceptible to bulb infection indirectly by increasing neck diameter (and possibly by influencing other plant parameters that were not monitored, such as leaf waxes and plant defences).

An increase in the number of irrigations on a crop can affect leaf wetness and increase humidity within the canopy, thereby increasing the potential infection periods, production of conidia and the splash dispersal of spores. Alderman and Lacy (1983) dusted dry conidia of *B. squamosa* onto onion leaves and found lesions were initiated after a minimum of 6 hours of dew. Kritzman *et al.* (1981) found that *B. allii* penetrated leaves at 93-100% RH but not at 80-85% or 70-75% RH. Snow (1949) reported that spores of *B. cinerea* required RH above 90% to germinate, and took 2 days to germinate at 93% compared to 1 day at 100% RH.

The environmental conditions that occur during growth of an onion crop can greatly influence *B. allii* epiphytotics (Maude and Presly, 1977a,b). The glasshouse (Chapter 9) and field trial experiments established with the same seed lot in this study have demonstrated that environmental conditions during seed germination can influence the transmission of infection of *B. allii* seed to seedlings. Weather parameters later during the crop growth also appear to affect the incidence of plants that become infected with *B. allii,* as demonstrated by the effect of the irrigation experiments in this study.

Anecdotal evidence suggests that high rates of nitrogen application can increase susceptibility of onion to *B. allii.* This may be due to the production of foliage that is more susceptible to infection compared to plants receiving less nitrogen. Alternatively or in addition, it may be due to production of thicker necks in onions receiving more nitrogen, which take longer to dry during the curing process, thereby increase the window of opportunity for *B. allii* to grow from leaves to bulbs. Unfortunately, *B. allii* leaf infection was not detected in 4700 leaf samples collected from the nitrogen

treatment area of this study. This occurred despite an application of inoculum to the nitrogen treatment Beds 6 and 7 on the 13/2/01 and the close proximity of the nitrogen trial to the infection bed, separated only by a 4 m-wide irrigator run planted to grass.

High concentrations of nitrogen in plants can increase their susceptibility to other diseases (Agrios, 1997). For example, *B. cinerea* is stimulated in grape to cause *Botrytis* bunch rot when nitrogen levels are high in the plants. Excess nitrogen causes excessive vegetative growth, which is more readily subject to physical damage and perhaps provide a more humid microclimate in the canopy for infection and sporulation of pathogens. However, a deficiency of nitrogen can also weaken plants, make them slow-growing and prone to age faster (Snoeijers *et al.*, 2000). For example, reduced availability of nitrogen increased the susceptibility of tomato to wilt caused by *Fusarium oxysporum* f.sp. *lycopersici,* early blight caused by *Alternaria solani* and damping-off of seedlings, caused by *Pythium* spp. (Agrios, 1997).

Wright (1993) concluded that onions that received excessive and late nitrogen applications, developed more foliage and thicker onion necks than onions that did not receive such nitrogen applications. The nitrogen treatments made the onions more prone to wounding during topping and to subsequent infection by soft-rotting bacteria (Wright, 1993). Kashi and Frodi (1998) stated that the increase in thickness of the bulb neck and width of the bulb were affected by nitrogen rate (applied as urea). They applied 0, 40, 80, 120, 160 or 200 kg N/ha and found the highest yield of onion and best total soluble solids (TSS) and dry weight (DW) occurred at a rate of 120 kg N/ha. At higher levels of N, the TSS and DW decreased. Applying more than 40 kg N/ha

had negative effects on the firmness of the bulbs and weight loss percentage during storage.

The higher nitrogen treatments and the high irrigation experiment in this study both suggested lower bulb diameter:neck diameter ratios, although not statistically different or tested, respectively. This indicated that proportionally larger onion necks occurred in the high nitrogen and irrigation experiment than in the corresponding low nitrogen and irrigation experiment. El Oksh *et al.* (1993) also noted that the bulb to neck ratio decreased with increasing nitrogen application (i.e., larger necks relative to increasing bulb size). Perhaps the ratio of bulb to neck diameter should be considered when devising nitrogen and irrigation management plans. Further work is required to investigate the effect of nitrogen and irrigation on susceptibility of onion to *B. allii* infection.

Chapter 9

9 Influence of benzimidazole fungicides on a *Botrytis allii* epiphytotic

9.1 Introduction

The relative role of seed and agronomic factors that affect epiphytotics of *B. allii* neck rot are not well understood. The experiments described in this chapter were aimed at gaining a better understanding of the seed-to-seedling transmission of *B. allii,* the efficacy and validity of current seed treatment practices used by the Tasmanian onion industry in disease management and the effect of fungicide treatments during the season on incidence of infection of plants by *B. allii.* Possible alternatives to the current seed treatment practices were also investigated.

9.1.1 Seed treatment efficacy

The current seed treatment practice by the Tasmanian Onion Industry is to use either a dusting of the fungicides Benlate®/Thiram® onto seed coated with wallpaper glue or Benlate®/Thiram® applied with a polymer. The seed is treated with these fungicides for control of *B. allii* and to prevent damping-off and other fungal and bacterial diseases. The efficacy of the two current seed treatments were examined for comparative purposes.

There is a high dependence on Benlate® (benomyl) as a control for *B. allii* in onion production in Tasmania. Benlate® is often used as a seed dressing and may be applied a further four times to the crop to control *B. allii* in the field. Prior to mother bulbs being planted in seed crop production, they are dipped in a Benlate® solution in an

attempt to ensure clean stock from which the seeds are produced. The seed crops may also be sprayed with Benlate® to prevent *B. allii* from causing scape blight and infecting the developing seed (C. Garland, South Pacific Seeds Pty. Ltd., Richmond, Tasmania, *personal communication*). The resistance of *B. allii* to Benlate® has been reported in the UK from onions originating from Holland (Gladders *et al.,* 1994), and has been reported in Israel (Kritzman, 1983b). Presly and Maude (1980) demonstrated how quickly Benlate® resistance can develop in *B. cinerea.* After three seasons in which Benlate® was used as a seed treatment to control *B. cinerea* on salad onions, isolates insensitive to the fungicide were detected.

9.1.2 Fungicide crop spray efficacy

Current industry practice for fungicidal control of *B. allii* is to apply Benlate at 1.0 kg/ha. In addition, Folicur (tebuconazole) is applied at 300 ml/ha for *Sclerotium cepivorum.* Fungicides are applied as two sprays, 7 days apart at the 4-5 leaf stage, followed by a further two applications, 7 – 10 days apart at the commencement of bulbing.

The objectives of the trial were to monitor the effects of benzimidazole fungicides on epiphytotics of *B. allii* in an onion crop, and to compare the incidence of *B. allii* in the leaves to incidence of *Botrytis* neck rot that developed in bulb samples after a storage period.

9.2 Materials and Methods

9.2.1 Seed test

The same seed line with a high incidence of *B. allii* was used in the seed treatment efficacy experiment in this chapter and in Field trial 2 (Chapter 8). The incidence of *B. allii* in the "infected" and "healthy" seed lots was ascertained by the methods described in Appendix 12.4. One thousand seeds were tested per line, with five seeds per Petri plate.

9.2.2 Glasshouse experiment to test efficacy of seed treatments for control of *B. allii*

The seed line with a high incidence of *B. allii* was used to test the efficacy of all seed treatments. A clean seed lot was used to provide a negative control (Treatment 1). Fungicide treatments were applied by D. Gilham (Field Fresh Tasmania Pty. Ltd.). The experiment was arranged as a randomised complete block design, with the 5 treatments (listed below), replicated 5 times. Aluminium trays (25 x 25 x 6 cm) with holes punched in the bases for drainage were used for seedling germination trays (1 tray per replicate and treatment i.e., 5 treatments x 5 replicates = 25 trays). Non-pasteurised red Ferrosol soil originally obtained from an old pasture was placed in each tray to a depth of 5cm. Lots (1.5g) of the seed from each of the treatments and replicate were weighed. At planting, 1.5g of seed per tray was scattered onto the surface of the soil and lightly mixed with the top 1 cm of soil. The various treatments are listed below (Table 9.1).

Treatment 1

Non-infected seed without fungicide treatment was used as a negative control to check for the spread of disease among treatments in the glasshouse. The clean seed was a regular cream gold line from Field Fresh Tasmania Pty. Ltd. (9-C-175), which was tested by the agricultural consulting company Serve-Ag Pty. Ltd. on the 8/5/00. A second sample was tested by the author on the 28/7/00 using the standard testing procedure (Appendix 12.4).

Treatment 2

This treatment consisted of a polymer coating technique to apply fungicide to the seed, and had been trialled by one of the local onion companies. Neck rot detected commercially during the season of implementation by the company suggested that this treatment may not have been efficacious against *B. allii*. However, the polymer coating technique of applying the fungicide to the seed was easier and safer than the standard commercial method used in Tasmania (described below) which used wallpaper glue and fungicide dusts. The same rates of fungicides were applied to the seed as in Treatment 3.

Treatment 3

This treatment involved dusting seed with the fungicide, and is the usual practice for the Tasmanian onion industry. Seed was first coated in a solution of wallpaper glue and then dusted with the fungicides (1 g Benlate® and 0.5 g Thiram® per kg seed) in a cement mixer.

Treatment 4

Hypochlorite treatment of seed was used as described by Metcalf (1998). Seed was soaked for 20 minutes in a 10% solution of sodium hypochlorite and then air dried in a laminar flow cabinet for 1 hour. Seed was sown 24 hours later.

Treatment 5

Infected seed without any fungicide seed treatments was also sown. This was the same seed lot as that used in Treatments 2-4.

Table 9.1: Seed treatments used in a glasshouse experiment to test for efficacy of *B. allii* control.

Treatment No.	Treatments	Seed line
1	Clean seed line, negative control	9-C-175 cream gold variety
2	Polymer coated Benlate and Thiram	Infected seed, cream gold variety
3	Dusted with Benlate and Thiram	"
4	Hypochlorite	"
5	No treatment, positive control	"

Trays planted with seed of the various treatments were arranged as a randomised complete block design on benches in a glasshouse. The trays were watered by hand once every 2 days using a water wand. Trays were sown on 1/8/00 and harvested on 18/9/00, 48 days later.

Seedlings were harvested at the first true-leaf stage when seed capsules were still attached to the cotyledons. Seedlings were placed into incubation trays (as described in Appendix 12.8), with each tray lined with a moistened tissue. Five seedlings were placed in each tray.

Sample assessment and analysis

A dissecting microscope was used to observe conidiophores of *B. allii* and *B. cinerea* on the seedlings. Seedlings were assessed twice, 9 and 17 days after beginning incubation of the seedlings in the trays, as not all of the seedlings had senesced by the first assessment date. Spores of suspect *Botrytis* spp. were placed onto Pectin agar (Appendix 12.5.1) if there was any doubt as to the species of *Botrytis* observed on the plant (Appendix 12.7). The effect of seed treatments was assessed by analysis of variance, with means comparisons using least significant difference (Genstat® v4.2).

Weather data

Air temperature, relative humidity, leaf wetness and rainfall were measured using a data logger (WatchDog model 450 data logger; Spectrum Technologies Inc, Illinois, USA). The data logger was fitted with internal temperature and humidity sensors and an external leaf wetness sensor (cat. #3666 gold plated) and rain gauge (cat. #36651 – capable of 1/100th inch increments with +/-4% accuracy).

9.2.3 Field trial establishment

This field trial was conducted in conjunction with an agricultural consulting company (Agronico Pty. Ltd., 175 Allport St, Leith, Tasmania) to test the efficacy of Benlate® and alternative benzimidazole fungicides against *B. allii*. The trial was established and run by Agronico and fungicide efficacy and residue data were collected as the basis for permit applications and registration for *Botrytis* control in onions for the Tasmanian onion industry.

The field trial was located in Forth, Devonport (Figure 9.1, Table 9.2). The trial area was previously uncropped pasture before being prepared for the onion crop. The trial site was at least 2 km from other onion crops. The soil was a red Ferrosol, typical of cropping soils of Northern Tasmania. Triple superphosphate fertiliser was banded with the seed and applications of N:P:K (14:16:11) were applied at 500 kg/ha on the 24/9/01 and 5/12/01.

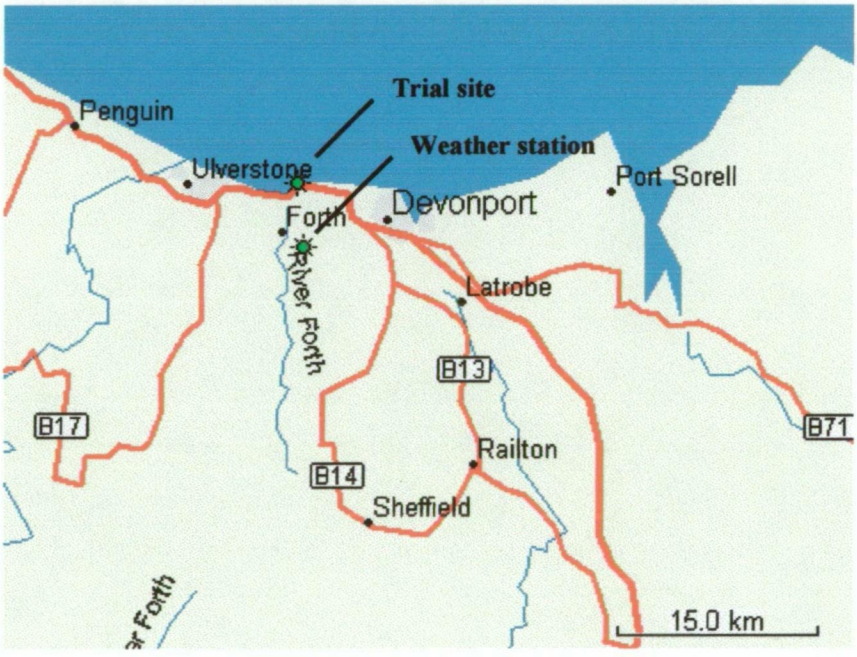

Figure 9.1: Location of field trial site and weather station.

Table 9.2: Coordinates of the trial site and weather station, using Universal Transverse Mercator position format and Australian geodetic 84-map datum.

Site	*Easting and northing*
Forthside weather station	55G 438200 5438300
Agronico Field Trial Site	55G 437700 5443100

The trial was sown on the 7/9/01. The total area was 15 m x 25 m, consisting of 9 beds (1.6 m wide from wheel centre to wheel centre) with 8 rows of onions per bed. The actual bed width was 1 m and beds were separated by 0.6 m. Seed of the onion cultivar 'Creamgold Regular' was sown with a precision drill by Field Fresh Tasmania Pty. Ltd. The seed did not have any fungicidal seed treatments applied. A seed test (Appendix 12.4) of 550 seeds found the seed to be free of *B. allii*.

9.2.4 Agronomic practices

A solid set micro-irrigation system was used to irrigate the trial. The jets were Philmac 360° microjet nozzles with 35 jets (5 rows of 7) set up approximately 3.5 m apart. Each jet irrigated a 3.5 m diameter area and jets were positioned so that water distribution from each nozzle overlapped slightly. Irrigation was applied as needed to avoid crop stress. The trial was irrigated a total of 5 times for an hour's duration each time. Herbicides and insecticides were applied to the plots as required (Table 9.3).

Table 9.3: Herbicides and insecticides applied to the trial.

Date	Chemical applied and rate	Control
6/8/01	Roundup[1]	burn off, pre-cultivation weed control
12/8/01	Ramrod[2] (10 L/ha)	burn off grasses
18/09/01	Basta[3] (3 L/ha) plus Stomp[4] (1 L/ha) and Lorsban[5] (0.7 L/ha)	weed burn plus insect control
1/10/01	Stomp (2 L/ha)	suppress weed emergence
15/10/01	Verdict[6] 520 (0.2 L/ha) plus Activator (0.1 L/100 L)	weed control
2/10/01	Tramat[7] (0.8 L/ha)	weeds
7/11/01	Totril[8] (0.5 L/ha) plus Tribunil[9] (0.5 g/ha)	weeds
13/11/01	Select[10] (0.5 L/ha) plus DC-Trate (40 mL/5 L)	weeds
23/11/01	Lorsban (0.7 L/ha)	insects
4/12/01	Totril (0.6 L/ha) plus Bladex[11] (0.6 L/ha)	weeds
2/01/02	Totril (0.75 L/ha) plus Tribunil (0.75 kg/ha)	weeds
15/01/02	Totril (0.75 L/ha) plus Bladex (0.75 L/ha)	weeds

[1]Glyphosate, [2]Propachlor, [3]Glufosinate-ammonium, [4]Pendimethalin, [5]Chlorpyrifos, [6]Haloxyfop-methyl (unstated stereochemistry), [7]Ethofumesate, [8]Ioxynil, [9]Methabenzthiazuron, [10]Clethodim, [11]Cyanazine.

9.2.5 Trial layout

The trial consisted of 9 beds sown in a north-south direction. Each bed was divided into 4 plots, and each plot was 5 m long. The trial was a randomised complete block design. Replicates of treatments were set up perpendicular to the beds (Figure 9.2), without buffers between treatments and replicates.

Bed	*Replicate 1*	*Replicate 2*	*Replicate 3*	*Replicate 4*
1	8	4	4	7
2	1	7	6	5
3	5	6	5	4
4	3	1	1	6
5	4	8	7	1
6	2	3	3	3
7	6	5	8	8
8	7	2	2	2
9	Non-inoculated bed			

Figure 9.2: Trial layout, demonstrating treatment and replicate locations.

9.2.6 Weather data

Weather data (air temperature, humidity, rainfall and wind speed) was collected from the Forthside weather station, located approximately 4 km south of the trial site (Figure 9.1).

9.2.7 Fungicide treatments

The three fungicides evaluated (Benlate®, Marvel® and Spinflo®) are all of the benzimidazole group (Table 9.4). There were seven fungicide treatments (Table 9.4) and one control treatment, with four replicates of all treatments. The fungicide applications were timed as per current commercial practice, i.e., 2 sprays applied one week apart at the 4-leaf stage (on the 5/12/01 and the 12/12/01), and 2 sprays applied one week apart at bulb initiation (on the 28/12/01 and the 4/1/02) (Table 9.5).

Table 9.4: Fungicide treatments, rates and corresponding treatment numbers.

Treatment	Fungicide and rate
1	Benlate (500 benomyl g/kg – Dupont) at 1 kg/ha (current commercial treatment)
2	Marvel (500 benomyl g/kg – Farmoz) at 0.5 kg/ha
3	Marvel (500 benomyl g/kg – Farmoz) at 1.0 kg/ha
4	Marvel (500 benomyl g/kg – Farmoz) at 2.0 kg/ha
5	Spinflo (500 carbendazim g/L – BASF) at 0.5 L/ha
6	Spinflo (500 carbendazim g/L – BASF) at 1.0 L/ha
7	Spinflo (500 carbendazim g/L – BASF) at 2.0 L/ha
8	Untreated control

Fungicide treatments were applied with a custom built PET (polyethylene terephthalate) bottle sprayer (Crop Care Australasia Pty Ltd, York, Western Australia). The sprayer had a 1.6 m boom with 4 Hardi 4110-12 flat fan nozzles. The PET spray unit was pressurised using liquid petroleum gas (propane/butane), and the plots were sprayed at 400 kpa pressure, with an output of 250 L/ha.

9.2.8 Inoculum

Two applications of *B. allii* inoculum were made to the whole trial except the untreated Bed 9 (Figure 9.2). The first application of inoculum was on the 22/11/01. Inoculum was prepared by growing isolate C3 of *B. allii* on PDA in petri plates, at 20°C for 10 days. Plates were flooded with sterile water and conidia scraped into suspension with a flat metal spatula. The spore suspension of approximately 1 x 10^5 spores per ml was applied to the plots using the PET sprayer described previously.

A second application of inoculum was made as a result of a low incidence of infection following the first inoculation. A spore suspension was made from three onions which had been artificially inoculated with *B. allii* (isolate C3) as described in Appendix

12.3. The total volume of spore suspension was 600 ml. The concentration of spores in the suspension was determined (using a haemocytometer) to be 3.34 x 10^5 spores per ml. The suspension was made up to 20 L and sprayed onto the crop on the 19/12/01, using the PET spray equipment as previously described. The rate of application was calculated as approximately 7.8 x 10^5 spores per m of onion bed. Assuming 65 plants per m^2, this equated to approximately 1.2 x 10^4 spores per plant. However, fewer spores were actually applied to the plants, as onions were at the 4 true-leaf stage when inoculated so the canopy was not completely closed.

An aliquot of the spore suspension was also plated onto 1% water agar in a Petri plate, and incubated in the dark at 21 °C for 24 hours. Spores were counted (at 200X magnification) to determine the percentage of viable spores. A germinated spore was defined as a spore with a germ tube equal in length, or longer than the spore.

9.2.9 Leaf sample collection

The percentage of plants infected with *B. allii* was determined during the trial by collecting leaves randomly from within plots 10 times over the course of the trial (Table 9.5). Leaf samples were not collected from the first and last 0.5 m of each plot, to provide buffering between treatments along beds. Twenty leaves were collected per plot for Treatments 1 and 8, as these were the treatments that were of most interest and 10 leaves were collected per plot for Treatments 2-7 due to time restrictions. Leaves were placed in food trays (5 leaves per tray) for incubation. After the 4th sampling, when leaves had become too large to fit easily into the trays, only 2 leaves were incubated per tray. Each tray was lined with a moistened tissue, and sealed with a lid after leaves were placed inside the trays. Trays were incubated at room

temperature (15-20°C) and examined after 7 days incubation for *B. allii* and *B. cinerea* conidiophores (Appendices 12.7 and 12.8). Leaf samples were not collected from the buffer bed (Bed 9) until the 16/1/02.

Table 9.5: Schedule of events for Field trial 3

Date	*Crop age (days)*	*DAI*[1]	*Event*
7/9/01	0		Seed sown
22/11/01	76		First spore suspension applied
23/11/01	77		Preliminary leaf sample of 40 leaves collected
5/12/01	89		1st fungicide treatment applied
12/12/01	96		2nd fungicide treatment applied
19/12/01	103	0	1st leaf sample collected
19/12/01	103	0	Second spore suspension applied[2]
26/12/01	110	7	2nd leaf sample collected
28/12/01	112	9	3rd fungicide treatment applied
2/1/02	117	14	3rd leaf sample collected
4/1/02	119	16	4th fungicide treatment applied
9/1/02	124	21	4th leaf sample collected
16/1/02	131	28	5th leaf sample collected
23/1/02	138	35	6th leaf sample collected
30/1/02	145	42	7th leaf sample collected
6/2/02	152	49	8th leaf sample collected
13/2/02	159	56	9th leaf sample collected
21/2/02	167	64	10th leaf sample collected
26/2/02	172	69	Onions lifted
20/3/02	194	91	Onions harvested and stored
30/5/02	265	162	Onion bulbs assessed for neck rot – 93 days after lifting

[1] Days after second inoculum of *B. allii* applied.

[2] Spore suspension was applied after the 1st leaf sample was collected.

9.2.10 Bulb collection and assessment for neck rot

The bulbs were hand lifted on the 26/2/02. Bulbs were field cured for 22 days. Approximately 1 kg (20-24 bulbs) per plot were collected and stored at ambient environmental conditions for 3 months in the laboratory. After storage, the bulbs were cut in half longitudinally and assessed for neck rot symptoms. *Botrytis allii* bulb infection was differentiated from other bulb rots by the characteristic symptoms and signs of the disease (Section 3.22).

9.2.11 Relationship between *B. allii* leaf infection and *Botrytis* bulb rot

For each treatment and replicate, the incidence of *B. allii* leaf infection for each sampling period was correlated with incidence of *Botrytis* bulb rot. The highest value of leaf infection that occurred during the sampling periods for each plot was correlated with *B. allii* leaf infection and *Botrytis* bulb rot. The relationship between leaf infection and bulb rot for all plots was also analysed using simple linear regression analysis, using Genstat® v. 4.2.

9.3 Results

9.3.1 Glasshouse seed treatment efficacy trial

The 'infected' seed lot was tested as per Appendix 12.4. The incidence of infection by *B. allii* was 28.9% ± 5.5% (standard deviation), derived from 5 replicate samples of 1000 seeds. *Botrytis allii* was not detected in seed lot 9-C-175, neither by the author nor by Serve-Ag Pty Ltd.

Conditions in the greenhouse trial were suitable for conidiophore development at the time seedlings were sampled as conidiophores were observed on the plants on 18/9/00 (48 days after sowing) (Table 9.6).

Table 9.6: Number of replicates in which conidiophores were visible on seedlings at the time of harvest (n=5)

Treatment	Clean	Polymer	Dusted	Hypochlorite	Infected
Number of replicates with *B. allii*	4	0	0	5	5

No significant differences in incidence of infected seedlings were found between the two assessment dates, and there was no significant interaction in the percentage of *B. allii* infection between the seed treatments and the seedling assessment dates. However, there were significant differences among the fungicide treatments ($P < 0.001$, $LSD_{0.05} = 10\%$) (Figure 9.3). Significant differences in incidence of infected plants were not found between the polymer coating and the dusting method of applying fungicide seed treatments, with 10.2% and 5.6% infected plants detected, respectively. The other three seed treatments were all statistically different, with 38.3%, 65.8% and 79.5% infected plants in the untreated clean seed, the hypochlorite treated seed and the untreated infected seed respectively.

No significant differences or interactions were found among treatments or assessment times in the incidence of plants infected with *B. cinerea.*

Cross contamination of *B. allii* between the treatments occurred in the glasshouse, as demonstrated by infection detected on plants in the uninfected seed line at sampling (Figure 9.3). Cross-contamination was also apparent in the untreated infected seed

line, as *B. allii* was detected in 28.9% of the seed tested but occurred in over 80% of the seedlings evaluated.

The incidences of infection that developed in the fungicide dusting treatment and the fungicide polymer treatment were low (Figure 9.3). The small amount of *B. allii* infection that did occur may have been from the infected seed or from cross-contamination from the other treatments involving infected seed. There was no significant difference between the two methods of fungicide application with respect to the percentage seedlings infected with *B. allii* or *B. cinerea.*

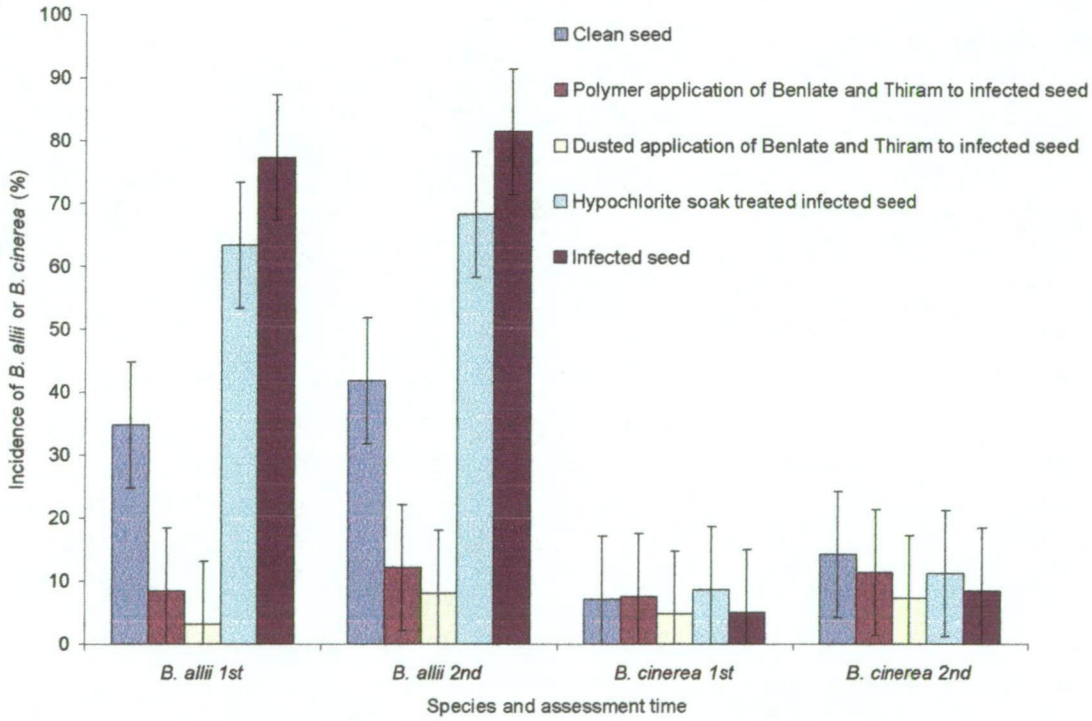

Figure 9.3: The effect of seed treatments on the incidence of *B. allii* in onion seedlings at 57 days after sowing (1st assessment) and 65 days after sowing (2nd assessment) (LSD=10 %).

9.3.2 Weather data collected for field trial

Rainfall was above the previous 7-year-average during the early part of the trial (September to November) and similar to the 7-year-average from December to January, in the latter part of the season (February and March) the rainfall was below the 7-year-average (Figure 9.4). In comparison, the 1995/96 season was a particularly conducive season for *B. allii*, with high incidences of neck rot in onion crops. This has been attributed to the high rainfall from January to March, a critical time for neck rot development and field curing (Dennis, 1996a). This indicated that the conditions during the field trial were not particularly conducive to bulb infection by *B. allii*.

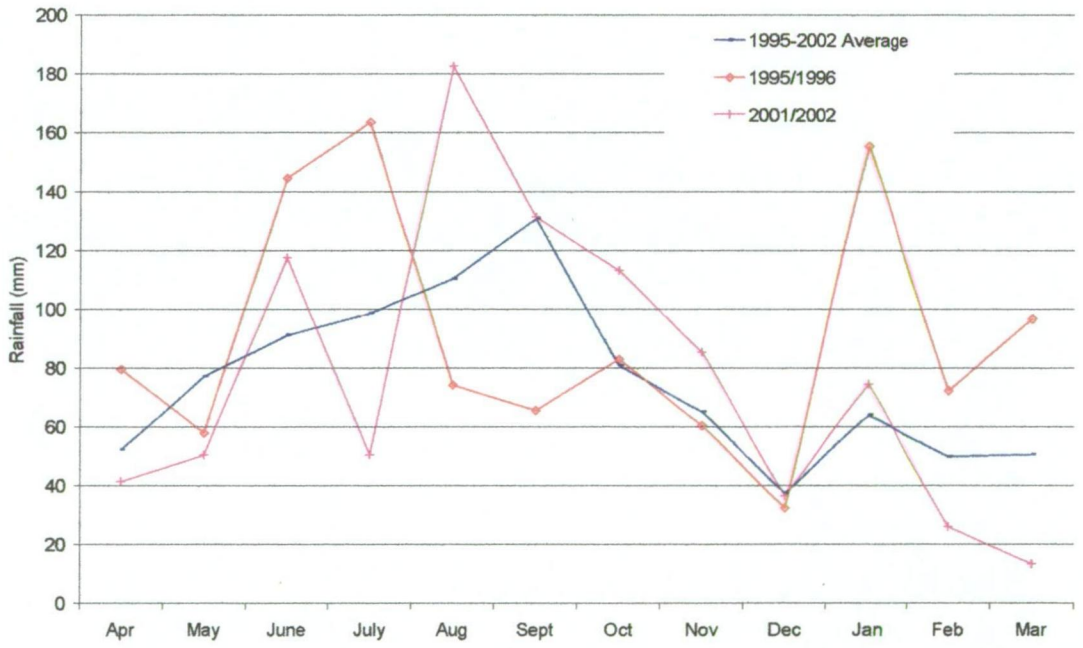

Figure 9.4: Rainfall recorded at the Forthside weather station.

9.3.3 *Botrytis allii* disease progress curve

The viability of spores in the first inoculum applied was not determined. In a preliminary sample of 40 leaves taken the day after application of the first inoculum (Table 9.5), only 1 leaf (2.5%) was infected. A subsequent sample of 360 leaves was

collected from the trial before application of the second inoculum (Figure 9.5) and no *B. allii*-infected leaves were found. The second inoculum was applied on 19/12/01. A suspension of spores incubated on water agar showed 91% germination of the spores in the inoculum (n=107).

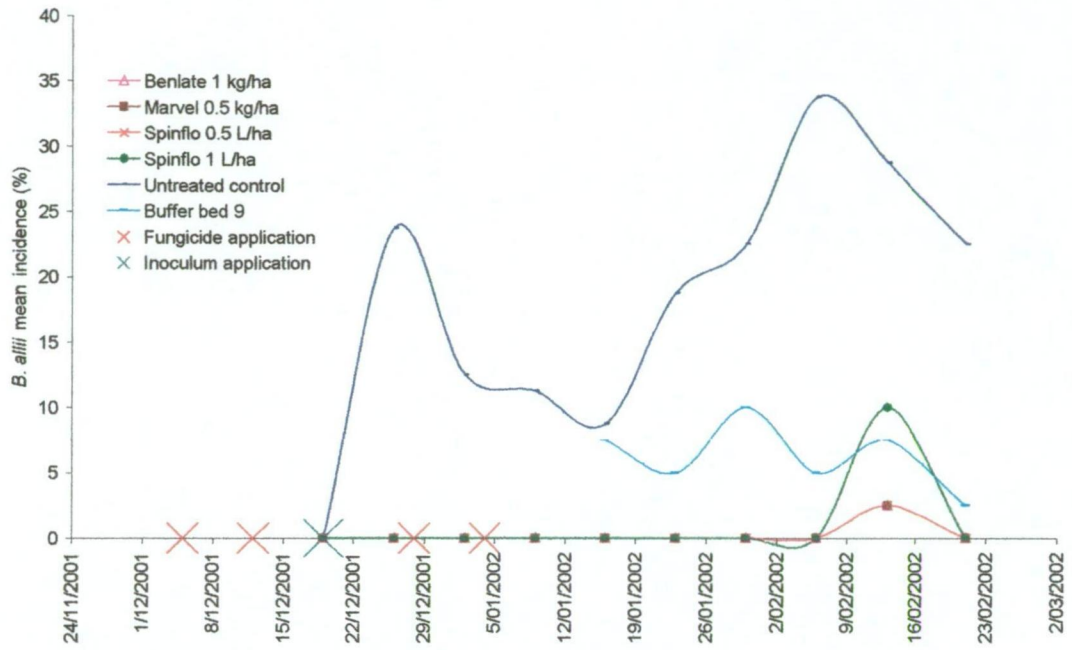

Figure 9.5: Disease progress curves for the average incidence of plants infected with *B. allii* each treatment. Benlate (1 kg/ha), Marvel (0.5 kg/ha) and Spinflo (0.5 l/ha) recorded the same incidence of *B. allii* on the 9th sampling (13/2/02) and the curves are overlaid on the chart.

The incidence of *B. allii* in the untreated control (Treatment 8) was significantly different among sample times (F-probability = 0.038, $LSD_{0.05}$ = 19) (Figure 9.5).

Botrytis allii was not detected in any of the leaf samples taken from plots Marvel® at 1 or 2 kg/ha or Spinflo® at 2 L/ha at any sampling period. Immediately after the second application of inoculum (19/12/01), *B. allii*-infected leaves were detected only in the untreated control plots (Figure 9.5). The incidence of leaf infection by *B. allii* in the untreated control plots rose to 23.8% at the second sampling time, before falling to

8.8% at the 5th sampling (16/1/02). The incidence of infection in the control treatment increased again after the 5th sampling to 33.8% at the 8th sampling (6/2/02), before falling to 22.5% in the 10th sample (21/2/02). The rate of increase in incidence of *B. allii* in the untreated control was 25% over 21 days (just > 1% /day) between the 16/1/02 and the 6/2/02. No leaf infection was found in any of the fungicide treatments until the 9th sampling, when 2.5% infection was found in plots receiving Benlate® at 1 kg/ha, Marvel® at 0.5 kg/ha and Spinflo® at 0.5 L/ha treatments, and 10% incidence in plots receiving Spinflo® at 1 L/ha (Figure 9.5). However, *B. allii* was not detected in these treatments at the tenth sampling.

The incidence of *B. allii* infection in Bed 9, which received neither fungicide applications nor inoculum, was 7.5%, 5.0%, 10.0%, 5.0%, 7.5% and 2.5% from the 5th to the final sampling, respectively (Figure 9.5). As Bed 9 was never sprayed with inoculum, the infection detected in this bed was most likely due to drift of inoculum or secondary spread of *B. allii* from infected plants in the untreated control plots.

9.3.4 Disease map

The highest incidence of infection occurring in any plot at any time is shown in Figure 9.6, and was examined to determine if there was any link between secondary spread of *B. allii* from infected (untreated) plots to neighbouring treated plots, once the protectant activity of the fungicides ceased. However, even though some fungicide treatments were located immediately adjacent to untreated plots, only low levels of leaf infection were detected in these plots, indicating fungicide treatments were effective even under conditions of high potential inoculum pressure. Some caution was required in data interpretation as the small number of samples collected from

some treatments probably contributed to variability in results between replicates and sampling periods.

Bed	Rep 1	Rep 2	Rep 3	Rep 4
1	45% (61.9%) T8	0% (0%) T4	0% (0%) T4	0% (0%) T7
2	5% (10.5%) T1	0% (4.2%) T7	0% (0%) T6	0% (0%) T5
3	10% (6.3%) T5	0% (4.2%) T6	0% (0%) T5	0% (4.3%) T4
4	0% (0%) T3	5% (0%) T1	0% (0%) T1	40% (0%) T6
5	0% (0%) T4	25% (42.1%) T8	0% (0%) T7	0% (0%) T1
6	0% (10%) T2	0% (4.2%) T3	0% (9.5%) T3	0% (18.2%) T3
7	0% (0%) T6	0% (0%) T5	40% (81%) T8	70% (68.4%) T8
8	0% (8.7%) T7	0% (0%) T2	10% (9.5%) T2	0% (20%) T2
9	10% B9	0% B9	10% B9	30% B9

Figure 9.6 Disease map showing the maximum incidence of plants infectd with *B. allii* in each quadrat over the sampling periods, the incidence of *Botrytis* bulb rot in parenthesis and the treatment number. T1 = Benlate at 1 kg/ha, T2 = Marvel at 0.5 kg/ha, T3 = Marvel at 1 kg/ha, T4 = Marvel at 2 kg/ha, T5 = Spinflo at 0.5 L/ha, T6 = Spinflo at 1 L/ha, T7 = Spinflo at 2 L/ha, T8 = untreated control, B9 = untreated and non-inoculated bed. Highlited plots demonstrate areas of nill fungicide treatments.

9.3.5 Bulb collection and assessment for neck rot

Neck rot due to *B. allii* was detected in bulbs sampled from many plots receiving fungicide treatments, ranging from 1.0% incidence in plots receiving Spinflo® (1 kg/ha) to 9.9% in plots sprayed with Marvel® (0.5 kg/ha) (Figure 9.6). There was no significant difference in mean incidence of neck rot between fungicide treatments. However, the incidence of *B. allii* neck rot in bulbs in the untreated control was

significantly higher than for all of the fungicide treatments (Table 9.7). Of the 32 plots assessed for neck rot only 16 plots (50%) contained bulbs with neck rot.

Table 9.7 : The effect of foliar fungicide treatments on the mean incidence of neck rot in bulbs

No.	Treatment and rate of application	Mean % infection
1	Benlate 1 kg/ha	2.6 a[1]
2	Marvel 0.5 kg/ha	9.9 a
3	Marvel 1 kg/ha	8.0 a
4	Marvel 2 kg/ha	1.1 a
5	Spinflo 0.5 L/ha	1.6 a
6	Spinflo 1 L/ha	1.0 a
7	Spinflo 2 L/ha	3.2 a
8	Untreated	63.4 b

Treatments means followed by different letters are significantly different (P=0.05, $LSD_{0.05}$=10.81)

9.3.6 Relationship between *B. allii* leaf infection and *Botrytis* bulb rot

The relationship between incidence of *B. allii* in leaves and the incidence of *Botrytis* neck rot that developed in bulbs after a storage period was significantly correlated at all sampling times except the first, when no infected leaves were found (Table 9.8). The number of plots that yielded infected leaves was low until the 8th and 9th sample periods, when 8 quadrats out of 32 contained infected leaves. Of the 32 quadrats in total only 16 quadrats contained bulbs with *Botrytis* bulb rot. Leaf sample period 2 (26/12/01) had the strongest correlation with the incidence of *Botrytis* bulb rot (r = 0.962) of all the sample periods, with a high R^2 value of 0.92 when fitted to a linear regression model (Table 9.8). The maximum incidence of *B. allii* found in the 32

quadrats was no better a predictor of the incidence of *Botrytis* bulb rot than the second, third, fifth or tenth leaf sample periods (Table 9.8).

Table 9.8: Correlation coefficient and linear regression analysis results (F- probability and R^2) between *B. allii* detected in leaves at sampling periods 1-10 and final *Botrytis* bulb rot in storage.

Leaf sample period	Correlation coefficient with bulb rot in storage	F-probability	R^2	# quadrats with incidence of infected leaves > 0 (of 32 total quadrats)
1	0	-	-	0
2	0.962	<0.001	0.92	4
3	0.876	<0.001	0.76	3
4	0.655	<0.001	0.41	2
5	0.857	<0.001	0.73	4
6	0.614	<0.001	0.36	3
7	0.765	<0.001	0.57	3
8	0.717	<0.001	0.50	8
9	0.717	<0.001	0.50	8
10	0.828	<0.001	0.68	4
Maximum[1] %	0.828	<0.001	0.66	9

[1] The maximum incidence of *B. allii* found in the leaf samples correlated with the final *Botrytis* bulb rot.

9.3.7 Botrytis cinerea

The incidence of plants infected with *B. cinerea* was low and uniform amongst the plots (Table 9.9). The incidence of *B. cinerea*-infected plants in the untreated control was not any higher (not statistically tested) than in the fungicide treated plots.

Table 9.9: Mean incidence (%) of *B. cinerea* leaf infection in the fungicide treatment trial (mean of 4 replicates per treatment).

Date of leaf sample period	Benlate at 1 kg/ha	Marvel at 0.5 kg/ha	Marvel at 1 kg/ha	Marvel at 2 kg/ha	Spinflo at 0.5 L/ha	Spinflo at 1 L/ha	Spinflo at 2 L/ha	Control	Buffer row
19/12/01	0	0	0	0	3	0	0	0	-
26/12/01	0	0	0	0	0	0	0	0	-
2/1/02	0	0	0	0	0	0	0	0	-
9/1/02	0	3	0	0	0	0	0	0	-
16/1/02	0	0	0	0	0	0	0	0	0
23/1/02	0	0	0	0	3	0	0	0	0
30/1/02	0	0	0	0	0	0	0	5	0
6/2/02	1	5	0	3	0	0	5	1	5
13/2/02	1	0	0	0	0	3	0	0	8
21/2/02	0	0	0	0	0	0	0	0	5

9.4 Discussion

9.4.1 Seed treatment efficacy

Conditions during the glasshouse experiment were favourable for *B. allii* conidiophore development and for infection of plants, as conidiophores were observed on the seedlings and cross-contamination occurred among treatments as demonstrated by the detection of *B. allii* infection on seedlings that grew from the clean seed. Thus, the levels of *B. allii* infection detected on seedlings that grew from treated seed were not just a result of what was existing on the infected seed but also of the protectant activity of the fungicides against subsequent foliar infection. Outright eradication of *B. allii* seed infection may have occurred, however as benomyl is systemic, the infections may have been managed by preventing seed transmission e.g. from the seed coat to the cotyledon.

Seedlings that grew from the hypochlorite treated seed had a high incidence of *B. allii,* significantly higher than that of the seedlings that grew from the 'clean' seed, suggesting this treatment was not effective at eliminating *B. allii* seed infection. If the hypochlorite treatment had eradicated *B. allii* from the seed then the incidence of infected seedlings would be expected to be similar to that of the clean line, with infection resulting from cross-contamination alone. It is generally considered that the majority of *B. allii* infection resides within the seed coat (Maude and Presly, 1977a; Stewart and Franicevic, 1994). Possibly, any external *B. allii* infection was removed by the hypochlorite, however internal infection may not have been eradicated. A test to distinguish between internal and external infection could be used to test this hypothesis.

The untreated seed lot used in the glasshouse experiment was the same as that used in Field trial 2 (Chapter 8). In Field trial 2, practically no infection was noted in leaves of seedlings sampled after emergence while in the glasshouse experiment a high incidence of infected seedlings was found. This indicates the significant influence of conditions during seedling emergence on the transfer of *B. allii* from seed-to-seedling. It is possible that wetter soil conditions in the seed trays compared to the field promoted seed-to-seedling transmission. The results also suggested that *B. allii* predominantly infects the cotyledon from the attached seed coat rather than by growing systemically from the seed embryo into the developing seedling. Systemic growth would be less affected by environmental conditions that that detected in this study.

Maude and Presly (1977b) reported that "it was apparent that although the benomyl seed treatment considerably reduced seed infection of *B. allii,* it did not eliminate it". They treated 10000 infected seeds (43% *B. allii*) with benomyl, and then sowed the seeds in compost in sealed plastic boxes (50 seeds/box). When the seedlings were 4 weeks old, they were removed and incubated on moist tissue pads in closed plastic containers for 5 days before being assessed for conidiophore production by *B. allii.* Eight infected seedlings were recovered (i.e., 0.08% infection), demonstrating that the fungicide treatment did not totally control seed infection or seed-to-seedling transmission as noted in field experiments and commercial crop surveys. The recovered *B. allii* isolates were found to be susceptible to benomyl (Maude and Presly, 1977b).

Fungicide resistant strains of *B. allii* have been reported in other countries (Maude and Presly, 1977b; Kritzman, 1983). Metcalf and Dennis (1998) found no Benlate® resistance in isolates of *B. allii* from Tasmania. The relatively low rate of *B. allii* seedling infection detected in this study from Benlate® treated seed suggested that good control is still offered by this seed treatment, particularly as it appeared that cross-contamination occurred among treatments in the glasshouse experiment and Benlate® continued to limit infection despite this. Presly and Maude (1980) used carbendazim treatment of seed to delay the onset of *B. cinerea* infections of salad onion seedlings by 9 weeks and 3 weeks in two different seasons. However, after using the carbendazim seed treatment for three seasons, isolates of *B. cinerea* were detected that were resistant to carbendazim, demonstrating that resistance can develop quickly in this genus.

To reduce the likelihood of seed-to-seedling transmission of *B. allii* in an onion crop, seed testing and treatment can be performed. Seed testing is used to determine the incidence of *B. allii* in the seed, so that a decision can be made as to accept (usually if the seed tests free of *B. allii* or the level is very low) or reject the seed lot. Treatment of seed with fungicides then reduces the seed transmission of any *B. allii* that may be present in the seed. Ensuring that these two steps are followed maximizes control of *B. allii* at the beginning of the crop season. The use of a seed treatment can greatly reduce the final incidence of bulb rot, by reducing the number of seedlings that become infected from an infected seed source (Maude and Presly, 1977b). Infected seed may be an important source of inoculum despite seed testing and seed treatment practices. The commercial crop surveys undertaken in this study have proven that infected seed is most likely still a source of infection in onion crops in Tasmania (Chapter 3) even though seed lots are routinely tested and treated with fungicides for *B. allii*. Furthermore, the exponential nature of *B. allii* epiphytotics, even in years of average rainfall (Field trials 1 and 2), highlights the need to reduce the initial level of *B. allii* as much as possible, to ensure the incidence of bulb rot remains at commercially-acceptable levels at harvest.

9.4.2 Seed treatment and potential for the future

Mebalds *et al.* (1997) investigated the use of steam-air treatments to control seedborne microbial pathogens of a range of vegetable and flower crops. They demonstrated complete elimination of *B. allii* from onion seed. The onion seed line used in the trial by Mebalds *et al.* (1997) had an incidence of 17.3% external and 6.8% internal infection by *B. allii*. Treatment of the seed at temperatures from 50 to 60°C (with 2°C intervals tested between these temperatures) for 30 minutes, was shown to remove *B.*

allii completely from the seed. However, *Aspergillus niger* was not eliminated from the seed lot at any of the temperatures evaluated, perhaps due to the thermophilic nature of this pathogen (Mebalds *et al.*, 1997). The germination capacity of the onion seed was not significantly affected by the steam-air treatments. This demonstrated that steam-air treatments may be used to control some fungal pathogens, in particular *B. allii,* without any negative effects on the seed.

Combining a physical treatment (e.g., steam-air treatment) with a fungicide seed treatment may enable greater control of seed-borne pathogens such as *B. allii.* This may also assist in preventing the build-up of fungicide resistant strains of pathogen, in particular of *B. allii* to the benzimidazole fungicides. A fungicide seed treatment would be necessary to prevent other plant pathogens present in the soil from damaging the seeds or seedlings. Tylkowska *et al.* (1998) found that a seed treatment of *Penicillium* spp. and *Trichoderma* spp. successfully controlled *B. allii.* In addition, this treatment was more effective against other seedborne fungi than the chemical treatment consisting of a mixture of benomyl, thiram and metalaxyl. However, treatment with *Penicillium* spp. also negatively influenced seed germination (Tylkowska *et al.*, 1998). Peach *et al.* (1994) reported that the bacterium *Enterobacter agglomerans,* isolated from neck rot-infected onions, was antagonistic to *B. allii.* A film coat application of the bacterium to seeds naturally infected by *B. allii* gave similar levels of control of the fungus to the standard benomyl seed treatment in laboratory tests and in stored bulbs produced from field-grown crops (Peach *et al.*, 1994).

9.4.3 Benzimidazole fungicide field trial

Benzimidazole fungicides were effective at controlling the *B. allii* epiphytotic. *Botrytis allii* was not detected in leaves sampled from plots receiving 3 of the 7 fungicide treatments at any time. In the 4 fungicide treatments in which *B. allii* was detected on leaf samples the fungus was not detected until 40 days after the 4th and final application of the fungicides. In contrast, an average of 28.8% of the leaf samples taken from the control plots were infected with *B. allii* at the 9th sampling. A low incidence of bulb infection occurred in some replicates in all fungicide treatments. However, the incidence of *Botrytis* bulb rot that developed in the untreated control plots was much higher than in any of the fungicide treatments. Some of the fungicide treatments were particularly effective, given that some fungicide-treated plots with low incidence of bulb rot bordered untreated plots with a high incidence of *B. allii.* In a commercial crop situation, such high disease pressure from within a paddock is unlikely as the entire paddock is usually sprayed with fungicides.

A reduction in the number of infected leaves detected in untreated plots between sampling periods 2 and 3 was noted. Perhaps the environmental conditions during this interval did not allow *B. allii* to produce secondary inoculum and infect newly emerging leaves, or to grow into new leaves as they developed. As leaves infected by the applied inoculum senesced, the incidence of infected plants may have declined until secondary spread occurred. Secondary spread of *B. allii* must have occurred in the trials, as there was a subsequent increase in the number of infected leaves detected in the untreated control plots. Furthermore, at the 9th sampling (13/2/02), 56 days after being challenged with the second *B. allii* inoculum, a low incidence of *B. allii* leaf infection was detected in some of the fungicide-treated plots. As was noted in

previous field trials (Chapters 7 and 8), the incidence of detectable *B. allii*-leaf infection declined as the crop matured and the tops began to lodge and the leaves began to senesce. It might be expected that the incidence of infection would keep increasing as the crop begins to senesce and sporulation of *B. allii* occurs. However, environmental conditions during this period may not always be conducive to sporulation and infection by *B. allii*. Furthermore, older senescing tissue may be less susceptible to infection by *B. allii* than young succulent tissue. In addition, at this stage of the crop life, saprophytic fungi may become more abundant in the senescing foliage and compete with *B. allii*. *Ulocladium atrum* has been demonstrated to inhibit *B. aclada* sporulation on dead onion leaves (Nielsen *et al.*, 2000; Kohl *et al.*, 1999; Kohl *et al.*, 1997; Kohl *et al.*, 1995). Kohl *et al.* (1997) noted that no direct parasitism of *B. allii* by *U. atrum* occurred, but *B. allii* was excluded completely from dead onion tissue when both of the fungi were competing for resources. Kohl *et al.* (1995) demonstrated that suppression of *Botrytis* sporulation can also be achieved by mechanically reducing the amount of necrotic leaf tissue available. It was concluded that suppression of sporulation on necrotic tissue is a valid control strategy, which could be applied using sporulation-suppressing antagonists (Kohl *et al.*, 1995).

On 26/12/01, *B. allii* was not detected in plots that had been treated with fungicides on 12/12/01 and inoculated with *B. allii* on 19/12/01. This suggested that the protectant activity of the fungicides lasted at least 7 days. In contrast, the untreated plots had a mean incidence of *B. allii* of 24% at 1 week after inoculation of the trial with *B. allii*. Evidence of the extent of protectant activity of the fungicides may also be found in the levels of secondary spread observed. It took 8 weeks from the time the trial was inoculated until secondary spread of *B. allii* was detected in some of the benomyl

treated plots. The duration of protectant activity of benomyl is unlikely to be this long as it would have taken time for inoculum produced for secondary spread to develop in the untreated plots. By the 6th sampling period, the incidence of *B. allii* detected had increased in most replicates of the untreated plots. Conversely, no infection was detected in the treated plots until the 9th sampling.

The first 4 leaf samples collected during the trial were comprised of leaves that were present when the inoculum was applied. However, leaves sampled in the 5th to the 10th sampling periods were not present at the time of application of inoculum. Therefore, these leaves must have been infected after secondary spread of *B. allii* within the crop or from systemic infection of the plants. Maude and Presly (1977a) demonstrated that *B. allii* moves within green leaf tissue. They suggested two types of plant infection. They stated that continuous infection throughout the whole plant, originates from infected seed and develops from the base of each successive leaf that develops. The other, more common, form of infection is the discontinuous infection of leaf tips with subsequent growth of the fungus down the leaves. The susceptibility of the leaf infected may also have affected the results, as young leaves with intact waxy layers may not be as susceptible to infection by *B. allii* as the older leaves with senescent tips and, possibly, insect damage. Kritzman *et al.* (1981) found that most of the leaf penetration by *B. allii* occurred in the 2-15 cm region of the leaf at the leaf tip. This region was approximately 10 times more susceptible than the leaf area 15-35 cm from the tip. Maude and Presly (1977a) also noticed that the leaf tips were infected by *B. allii* more often than other parts of the plant. Neither researcher explained the reason for this phenomenon. Leaf tips typically become necrotic in onion crops. There may be an association between increased infection of leaf tips by *B. allii* and the presence

of thinner or damaged leaf waxes at these sites as the leaves mature. Verity *et al.* (1981) noted a progressive increase in the amount of structured crystalline wax on successively produced (younger) onion leaves, with higher contact angles between water droplets and the leaf surface on the younger leaves, resulting in lower spray retention compared to older leaves. Verity *et al.* (1981) made no mention of variation in wax layer on individual leaves.

MacManus (2000) demonstrated the alteration of onion leaf-surface waxes by wetting agents used in pesticide applications. Such wetters may predispose onions to *B. allii* infection by reducing the waxy leaf barrier, facilitating penetration by *B. allii* and/or allowing water to sit on the waxy surface rather than run off the leaf (Metcalf *et al.*, 2001). Onion downy mildew is routinely controlled with fungicides which are applied on a weekly basis. No fungicides were applied to this crop other than the experimental treatments. It is possible that if the field trial had been sprayed on a regular basis for onion downy mildew, the crop may have been more susceptible to *B. allii* infection due to the alteration of leaf-surface waxes. Further experiments are required to determine the effect of leaf wetters on the *B. allii* epiphytotic.

Botrytis allii was found at a low incidence in the uninoculated Bed 9, as early as the first sample period (16/1/02). This indicated secondary spread had occurred from untreated plots or that infection had occurred as a result of spray drift of the inoculum during application. The incidence of *B. allii* in this bed remained below 10% from the 5th to 10th sample periods and did not increase, unlike what occurred in the inoculated non-fungicide treated plots. Visual observation suggested that the plants were growing poorly in Bed 9 compared to other beds of the experiment. Perhaps these plants were

deficient in nitrogen and thus were less susceptible to infection resulting in little increase in the incidence of *B. allii*. Wright (1993) found that levels of bacterial soft rot of onions increased with a high level of nitrogen, although nitrogen limiting environments have also been demonstrated to be conducive to disease development (Snoeijers *et al.*, 2000). Perhaps the smaller leaf area of these plants compared to more vigorous plants resulted in less accumulation of *B. allii* spores compared to other plots. A sparse canopy would also dry quicker and not provide as many leaf wetness periods for infection compared to a dense canopy, thereby reducing sporulation of *B. allii*.

The shape of the disease progress curve in the untreated plots (Treatment 8) did not visually fit any standard models of plant disease epiphytotics, unlike the disease progress curves for previous field trials (Chapters 7 and 8). The smaller number of samples collected in this trial compared to previous trials may have resulted in greater variation in the data, which may have affected the shapes of the curves.

The incidence of *B. allii* in the leaf samples collected and the incidence of neck rot that developed in stored bulbs was significantly correlated. The strongest correlation was between the second leaf sampling period and the incidence of *Botrytis* bulb rot ($r = 0.962$), with a high R^2 value (0.92) when fitted to a linear regression model. This supported the results of Chapters 7 and 8 that, under controlled field conditions, there is a significant relationship between leaf infection detected during the season and bulb rot detected after harvest. Maude *et al.* (1985) attempted to correlate weather factors with neck rot in bulbs over a 12 week growing season. The correlation between the log (% neck rot) and relative humidity from the 19th to the 26th of June for all years

from 1973-1980 was significant ($P<0.05$) with $r = 0.97$ (Pearson's correlation coefficient). It was assumed that the particular leaf growth stage at that time of year, and the presence of the leaves from this growth stage in the composition of the onion neck tissue at harvest may have accounted for the strong, positive relationship between these factors. Further experiments are needed to examine in greater detail the relationship between leaf and bulb infection.

Experimental work examining the effect of timing of fungicide applications on efficacy against *B. allii* may be beneficial in improving the current fungicide protocol recommended for *B. allii*. Ahmed *et al.* (1991) found benomyl or procymidone applied 4 weeks before harvest at a rate of 2 g/litre provided the greatest increase in yield and decrease in incidence of *B. allii*, compared to benomyl or procymidone treatment of transplants by dipping into the fungicide solutions (benomyl at 2g per L and procymidone at 20g per L). Koriem *et al.* (1991) tested benomyl, iprodione, vinclozolin and procymidone against *B. allii* in the laboratory and field. In the laboratory, sporulation and growth of *B. allii* was inhibited by 0.5 ppm benomyl and by 10 ppm procymidone or vinclozolin, while 10 ppm. iprodione had no effect on growth and increased sporulation of *B. allii* (Koriem *et al.*, 1991). The most effective disease control in a seed crop was provided by dipping onion sets in benomyl before planting, and iprodione was the least effective fungicide for control of neck rot (Koriem *et al.*, 1991).

The trial described in this chapter demonstrated that a high level of control, (although not necessarily a commercially acceptable level of control of *B. allii* can be achieved using any of the treatments tested (including the current industry practice of 4

applications of Benlate® mentioned above Section 9.1.2). Good control of neck rot occurred even when the onion plants were challenged with relatively high inoculum pressure that resulted in a mean incidence of 63% neck rot in bulbs from the untreated control plots. Use of the benzimidazole sprays up to 118 days after sowing did not result in any significant fungicide residue levels in bulbs at harvest (J. Hills, *personal communication*).

Chapter 10

10 General conclusions and suggestions for future research

It has recently been established, using molecular methods, that there are three predominant neck rot species. *Botrytis aclada* type AI, *B. aclada* type AII (*B. allii*) and *B. byssoidea* (Nielsen and Yohalem, 2001). The differences in spore sizes between *B. aclada* types AI and AII demonstrate that there are biological differences between the two types. Now that molecular methods are available for the detection and differentiation of neck rot species, further studies can be undertaken using these molecular tools to understand the epidemiological differences among them. The survey in this study of a limited number of Australian isolates of neck rot fungi suggested that *B. allii* (*B. aclada* type AII) was the dominant species causing neck rot in Australia. Further studies looking at more isolates would be required to verify if this is correct, and if so, studies investigating the differences in biology and epidemiology may provide answers as to why this is so.

The difficulty in detecting *B. allii* infection in the field makes field studies laborious and time-consuming, as demonstrated in the survey of commercial crops described in Chapter 3. The survey demonstrated that *B. allii* was present in a number of crops, and is likely to be present in most onion crops early in the season, but undetectable using current methods of detection. The size of commercial onion crops in Tasmania and the number of plants contained therein means that careful consideration must be given to the sample size and sampling procedure to obtain a representative sample of

onion plants. For example, if it is necessary to detect low levels (less than 1%) of *B. allii* with certainty then large sample numbers would need to be collected over the entire paddock. The exponential nature of epiphytotics which can mean that very small quantities of initial inoculum can lead to commercially unacceptable incidence at harvest For example, 3.6% neck rot was detected after 150 days exposure to a cull pile of 12 onions; and 6.1% and 3.1% after 43 days exposure to an artificial spore inoculum source in high and low irrigation applications respectively), as described in Chapters 6, 7 and 8. Some good correlations were obtained between leaf samples collected early in the season and subsequent bulb rot after storage in some experiments in this study. However, the amount of sampling required would suggest that this technique is likely to be commercially prohibitive as a means of forecasting bulb rot in storage. Given the exponential nature of epiphytotics it is unlikely that an early season test of sufficient sensitivity could be developed to detect the low levels of initial inoculum which can lead to commercially unacceptable incidence of bulb rot after harvest. Furthermore, in years where secondary spread occurred during the crop life, sampling of leaves early in the season would give a poor representation of the incidence of infected plants at harvest. The steepness of *B. allii* disease gradients (i.e. gradients tend to fall sharply within 5 m from a foci of infection) and aggregated spatial distribution may also make it difficult to obtain a representative sample from a field/crop, as infection foci tend to be discrete and localised.

Detection of spores of *B. allii* is difficult due to size and indistinct nature of the spores. The experiments conducted in this thesis demonstrated that spores are spread both during rainfall events and during periods without rainfall. The inoculum and disease gradients observed (Chapters 7 to 9) demonstrated that the predominant

spread within a crop was short-range (predominantly within 5m of source), which was likely due to successful deposition of living spores and subsequent plant infection associated with rainfall events. Although the importance of long-range aerial spread of *B. allii* was difficult to quantify, it should not be overlooked. The use of molecular and immunological techniques would assist greatly in understanding the spore dispersal and plant infection process of *B. allii*, particularly with spore trapping as such techniques may reduce the time required to identify spores. A specific antibody or PCR primer for *B. allii* would assist with/compliment visual identification of the pathogen.

The detection of latent *B. allii* infections in bulbs is important for the onion bulb export industry and the seed production industry. The bulb tissue sampling and incubation procedure evaluated in this study may provide a means of testing bulbs routinely, although it is a time-consuming process (Chapter 4). The development of an accurate, precise and rapid test for the detection of latent infections would be of benefit to the export industry, by providing a guarantee of a low incidence of infection. Such a test would also be of use in the selection of mother bulbs for seed production, reducing the incidence of *B. allii* within seed crops. Methods of detecting *B. allii* prior to storage other than by incubation, include immunological and molecular-based methods. Nielsen *et al.* (2002) have reported good results using a PCR assay with primers specific to *Botrytis* spp. for detection of infected leaf tissue and bulbs. This technique allowed more rapid detection of latent infection than incubation. Better detection of latent infections using molecular and immunological methods may be obtainable in comparison to visual techniques, which require an extended incubation period of 7 days or more, as a percentage of *B. allii* tissue

infections may be overrun by saprophytes. However, even with immunological and molecular detection systems, sampling procedure and choice of tissue to assay will still be critical factors in the successful commercial use of these methods. Furthermore, pre-incubation of tissue may still be necessary to provide detectable levels of infection.

Onion seed is currently tested by plating seeds onto agar in Petri plates followed by an incubation period to encourage *B. allii* growth onto the agar if present in the seed. Currently 550 seeds are tested per seed lot by the Department of Primary Industries Water and Environment. If a negative result is returned, this sample size gives 99% confidence that there is no more than 1% incidence of *B. allii* in the seed lot. To achieve 99% confidence that there is no more than 0.1% *B. allii* in the seed lot, 5000 seeds would need to be tested (Johnston and Kotz, 1969). With the current seed testing protocol (Appendix 12.4), this level of sampling would be very time-consuming. Assuming there are about 650,000 plants/ha in an onion crop and if 0.1% of the seed planted are infected (below the current detection level), and assuming favourable conditions occur for transmission of *B. allii* from the seeds to the plants, this will result in 650 infected plants/ha at the beginning of the growing season, with the possibility of an exponential increase in incidence towards the end of the season as observed in field trials. The development of either an ELISA or molecular detection method for *B. allii* in seed may allow for the routine testing of adequate seed samples, without the week-long incubation period needed for the agar assay.

Seed production needs to be managed carefully, as this is potentially a significant source of *B. allii* for bulb crops. The areas where onion seed are grown should be

chosen carefully. Locations that are isolated from other commercial *Allium* crops and that have dry climate should be chosen, where possible. Treatment to eliminate or reduce *B. allii* infection of mother bulbs or seed needs to be performed to prevent *B. allii* establishing in the seed crop.

Given a larger budget, immunological detection of *B. allii* would have been pursued in this study using monoclonal antibodies for the detection of *B. allii* propagules. Monoclonal antibodies (Mab) offer greater specificity than polyclonal antibodies (Pab's) and have been developed for other *Botrytis* spp. (Meyer *et al.*, 2000), but are not as yet available for *B. allii.* Now that molecular methods for detection of *B. allii* are available, these too may be used for more accurate, rapid and sensitive detection of *B. allii.* The use of molecular and immunological techniques will likely enhance the collection of epidemiological data on *B. allii.* Perhaps in the future monitoring techniques based on these methods may enable more effective control strategies to be implemented for *B. allii.* One possible detection system that is currently being developed for fungal pathogen systems is the microtitre immunospore-trapping device (MTIST) (Kennedy *et al.*, 2000). The MTIST traps fungal spores by impaction in microtitre wells. The enzyme linked immunosorbent assay (ELISA) technique can then be used to quantify target spores, given an appropriate specific antibody is available (Kennedy *et al.*, 2000). An MTIST system would be invaluable for research and, once developed, could be used strategically to apply fungicides when *B. allii* spores are released and environmental conditions are conducive for infection.

The duration of survival of *B. allii* in soil was found to be much longer under Tasmanian conditions than in the United Kingdom by Maude *et al.* (1982).

Consideration should be given to this in devising management plans. Neighbouring fields should also be included in the management plans due to the possibility of sclerotial germination and spread of conidiophores to neighbouring fields. Additional survival studies could examine the survival of *B. allii* on crop trash, as this may extend the overwintering capabilities of *B. allii*. The conditions that trigger sporulation or prolong sclerotial longevity would also be worth investigating, as management practices such as irrigation or other cultural practices may play a role in reducing sclerotial survival. Alternative hosts may also assist *B. allii* in overwintering, and in spreading to other onion crops. The role of alternative *Allium* weed hosts, which are present in Tasmania, as well as potential for non-*Allium* hosts, needs investigation.

The current fungicide management protocol used in onion production in Tasmania was demonstrated to reduce the incidence of neck rot in storage. However, improvements could be made in the timing of fungicide application and in screening for alternatives to benzimidazole products to reduce the chance of fungicide resistance developing. The strategic application of fungicides for control of neck rot may be possible if a *B. allii* forecasting model is developed, such as those produced for *B. squamosa* and *B. cinerea*. The interaction of leaf wetters used with fungicide applications, and the promotion of extended leaf wetness and removal of the protective layer of leaf waxes, may play a role in *B. allii* epiphytotics and warrants further investigation.

This thesis has provided insight into various epidemiological aspects of the *B. allii* life cycle, from the start of an epidemic associated with seed infection, to plant infection

and spread of inoculum, to the survival of propagules in soil. The results of this study will assist in more effective management of neck rot and in identifying/defining future research on this pathogen.

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Wu, B.M., A.H.C. van Bruggen, K.V. Subbarao, and G.G.H. Pennings. 2001. Spatial analysis of lettuce downy mildew using geostatistics and geographic information systems. *Phytopathology* 91:134-142.

12 Appendices

12.1 List of publications and presentations as a result of the study

12.1.1 Posters

Chilvers, M.I., Hay, F.S., Wilson, C.R. and Metcalf, D.A. 2001. Epidemiology of *Botrytis allii* (syn. *B. aclada*) – onion neck rot. Phytopathology 91(6):S17.

Chilvers, M.I., Dennis, J.J., Hay, F.S., Wilson, C.R. and Metcalf, D.A. 2001. Detection of latent *Botrytis allii* (syn. *B. aclada*) infections in onion bulbs. Phytopathology 91(6): S17.

Metcalf, D., Dennis, J. and Chilvers, M. 1999. Epidemiology and control of *Botrytis allii* in Tasmanian onions. Conference proceedings, 12th Biennial Conference, Australasian Plant Pathology Society, Canberra, pg 326.

12.1.2 Industry articles

Chilvers, M. 2001. Unravelling the epidemiology of *Botrytis allii*. Onions Australia 18:5-7.

Chilvers, M. 1999. Botrytis in the 90's. Onions Australia. 16:28-30.

12.2 Spore solution

Spore solutions of *B. allii* were made either from cultures growing on agar or from sporulating bulb material, and where necessary spore counts were performed with a haemocytometer.

A 1 x 1 cm^2 section of sporulating culture was cut from the pectin agar or from the sporulating bulb or bulb half piece and transferred to a 10 ml McCartney bottle or tissue culture jar. After adding a drop of Tween 80 and the appropriate amount of dH_2O, the mixture was shaken vigorously by hand or with a vortex mixer for approximately 1 minute.

12.3 Onion bulb inoculation by injection and inoculum production

Onion bulbs were artificially inoculated for the production of inoculum. A spore suspension was produced as previously described (Section 12.2). A needle was used to puncture the onion neck at an angle towards the centre of the bulb, to penetrate as many scales as possible. This first needle puncture prevented a second needle inserted into the bulb from clogging. A 0.5-1.0 ml aliquot of spore suspension was then injected into the neck of the bulb using the second needle and syringe. Neck rot symptoms developed in the inoculated bulbs within 2 weeks. If large amounts of conidia were required, then the bulbs were cut in half longitudinally 1 to 2 weeks before use, to provide a larger surface area on which the fungus could sporulate.

12.4 Seed testing

Taken from: Metcalf and Dennis (1998)

1. Weigh 3.0 g of onion seed and place in a tea strainer or similar wire mesh device.
2. Measure temperature of the 3% sodium hypochlorite solution and deionised water:
3. 22°C has been used as a standard. It is possible that warmer liquid could eradicate *B. allii* from the seed.
4. Immerse seed in seed strainer in 80 ml of 3% sodium hypochlorite for 1 minute.

5. Immerse seed in strainer in 2 changes of 100 ml sterile de-ionised water for 1 minute each.
6. Empty seed from strainer into sterile Petri dish or similar vessel.
7. Using tweezers, place seeds individually on pectin agar (25 seeds per plate). Tweezers should be dipped in alcohol and flame sterilised after every five seeds. Tweezers should not be allowed to become hot, as this could eradicate *B. allii* from the seed.
8. Incubate seed at 25°C for 7 days (it may be necessary to mark colony origin at 5 days) before identification of *Botrytis* colonies.

All sterilisation and plating procedures were performed within the laminar flow cabinet. Sodium hypochlorite should be stored under refrigeration to avoid degradation and not used more than 6 months beyond the date of purchase.

12.5 Agar recipes

12.5.1 Pectin agar

Pectin agar is composed of (per litre):

-$NH_4H_2PO_4$	0.9 g
-$(NH_4)_2HPO_4$	2.0 g
-$MgSO_4.7H_2O$	0.1 g
-KCl	0.5 g
-Citrus Pectin	10 g
-Agar	30 g

Place all ingredients in distilled water in blender for 1-2 minutes (until pectin dissolves). Adjust pectin agar to pH 4.0 using HCl before autoclaving.

12.5.2 Kritzman's (modified) agar

Adapted from Kritzman and Netzer (1978).

Basal medium (per litre):

$NaNO_3$	1.0 g
K2HPO4	0.9 g
MgSO4.7H2O	0.2 g
KCl	0.15 g
Glucose	20.0 g
Agar	25.0 g

Autoclave and cool to 70°C, then add:

CuSO4.hydrated	1.7 g	add direct
tannic acid	5.0 g	dissolve first in water

Shake well as tannic acid tends to precipitate the media.

Then adjust pH to 6.0 with 1 N NaOH (Use ~23 ml of 1 N NaOH)

Then add following just before pouring (when at 50°C).

PCNB (99%)	0.005 g	1 ml of 0.05 g in 10 ml (acetone)
Dithane	0.0004 g	0.2 ml of 0.02 g in 10 ml (water)
Chloramphenicol	0.025 g	dissolve in ethanol and water

12.5.3 Half strength lactic acid potato dextrose agar

For 500 ml:

Potato Dextrose Agar (Oxoid)	9.75 g
Agar	2.5 g
Distilled H_2O	up to 500 ml
Lactic acid	0.7 ml (add after autoclaving, when temperature has fallen to 50°C)

12.5.4 Water agar

Water agar (WA) was made by adding agar to distilled water, at the concentrations required, i.e., for 0.5% WA, add 5 g of agar to 1000 ml of distilled water. Media was autoclaved.

12.5.5 Onion leaf extract agar

Per litre of distilled water:

Dried onion leaves broken into small pieces (5 mm – 10 mm)	15 g
Agar	16 g

Media was autoclaved.

12.6 Spore trapping and nuclei staining protocol

12.6.1 Trapping surface preparation

Coat slide or trapping disk/surface with Vaseline thinned in hexane (1:1), using paint brush. If necessary, heat the trapping surface with a hot air gun to melt Vaseline and ensure a smooth even surface.

12.6.2 Staining procedure (Adapted from Shirane *et al.*, 1989)

1. Place specimen in fixer 15 minutes.
2. 95% methylated spirits wash.
3. 70% methylated spirit soak (can be left overnight).
4. Immerse in 5 M HCl for 30 min.
5. Wash with dH2O.
6. Stain with Giemsa for 3 hr [prepare fresh from stock each time].
7. Rinse with dH2O to remove excess stain.

8. Place coverslip on specimen to observe – makes it easier to see spores, mount in water.

Fixer

3:1 methylated spirits : glacial acetic acid

Giemsa

Use 10 ml Giemsa stock in 150 ml Phosphate buffer pH 7.0

Giemsa stock

1.95 g Giemsa powder in 125 ml methyl alcohol and 125 ml glycerol

Note: Mounting in lactic acid removes Giemsa stain, as does glycerol. Water seems to be the safest mount – though not for long term storage of mounts.

12.7 Differentiation of *B. allii* and *B. cinerea* in culture and leaf tissue

Isolations onto Pectin agar or Onion leaf extract agar were performed on various *Botrytis* cultures to determine which species was present by cultural characteristics (Figure 12.1). *Botrytis allii* produces short (usually about 1 mm) conidiophores with occasional branches. *Botrytis cinerea* produces much longer (frequently 2 mm or longer) conidiophores with long branches. Conidiophores of *B. allii* are usually form a grey mass on Pectin agar plates, and on Onion leaf extract agar the grey mass of conidiophore is generally restricted to the pieces of onion leaf within the agar. *Botrytis cinerea* produces tufts of conidiophores in sparse patches across Onion leaf extract agar, with a similar appearance on Pectin agar but with more mycelial mat across the agar surface. *Botrytis allii* occasionally forms sclerotia on the two media

after a long period. However, *B. cinerea* will develop sclerotia rapidly on the two media. Under uniform laboratory conditions of leaf senescence, *B. allii* and *B. cinerea* conidiophores could reliably be differentiated by eye (Figure 12.1).

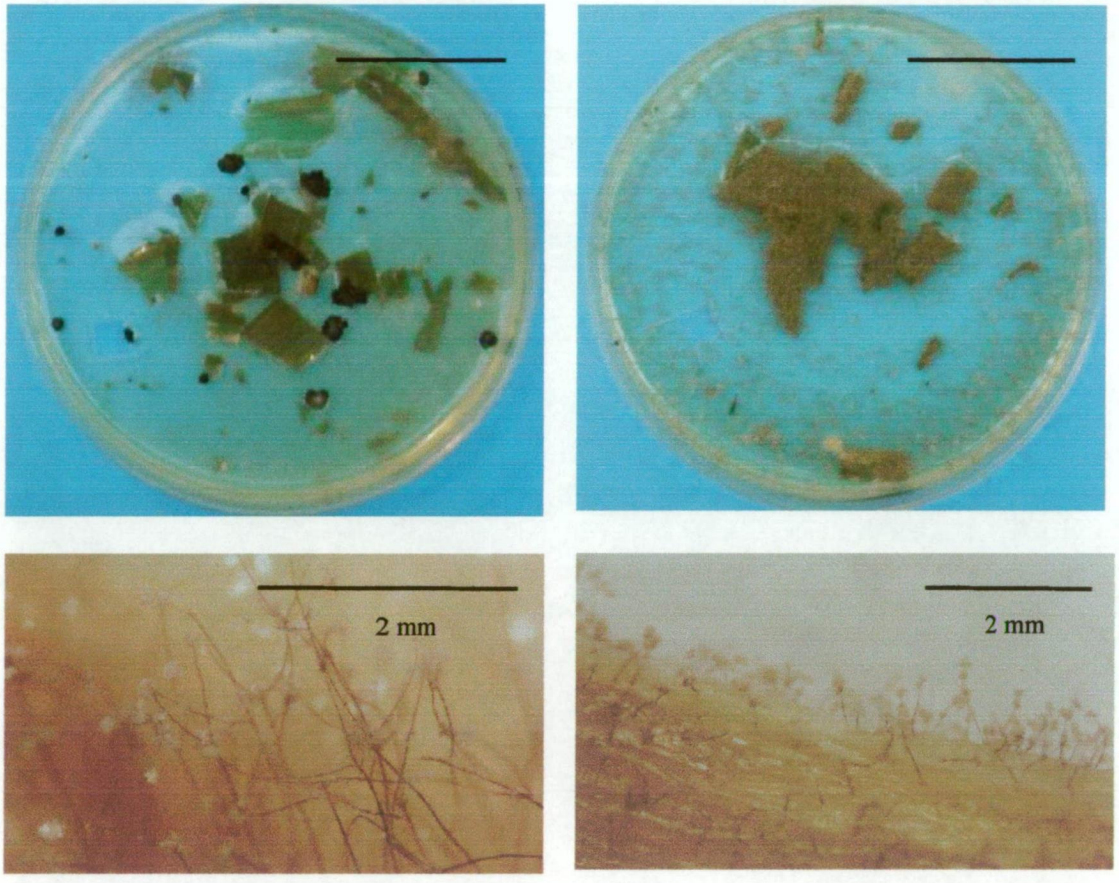

Figure 12.1: Onion leaf extract agar in Petri plates with *B. allii* (top right) and *B. cinerea* (top left). Conidiophores of *Botrytis* spp. on senescent leaf tissue: *B. cinerea* (bottom left – 18X magnification) and *B. allii* (bottom right - 10X magnification).

12.8 Incubation of plant tissue protocol

Plant tissue samples for incubation were placed into a non-sterile 1 L Genfac® plastic food tray (length 16 cm, breadth 10 cm and height 6 cm), lined with a moistened tissue and sealed with a lid. The trays were incubated at room temperature (15°C to 20°C) on the laboratory bench for 7 days to allow the plant tissue to senesce. The incubated tissue was observed with bright light and a dissecting microscope (X 40 magnification) for the presence of *Botrytis* conidiophores.

12.9 Isolate records

Isolate	Morphology	Identification based on DNA confirmation	Origin
C3	*B. aclada*	*B. aclada* (AII) (syn. *B. allii*)	HMR seed test 1999 (3/3/1999)
C4	*B. aclada*	*B. aclada* (AII) (syn. *B. allii*)	Metcalf's isolate collection - B1
C26	*B. aclada*	*B. aclada* (AII) (syn. *B. allii*)	Isolated from infected onion plant below soil line from Scottsdale (25/11/1999)
C29	*B. cinerea*	-	HMA9 seed test 1999 - seed treatment experiment
C30	*B. aclada*	*B. aclada* (AII) (syn. *B. allii*)	HMA9 seed test 1999 - seed treatment experiment

12.10 Field trial 2 records

Field Fresh Tasmania
Soil Analysis Report

CLIENTS NAME:	Burnie TAFE	SAMPLE DATE:	Jul-00
ADDRESS :	Mooreville Rd	LOCATION:	Burnie
PADDOCK NAME:	Trial Plot	AREA (Ha):	0.3
SOIL TYPE:	Krasnozem		
PREVIOUS CROP:	Pasture	CROP TO BE GROWN:	Onions

RESULTS: (Allison Laboratories Hobart)

	Your Levels	Recommended Levels	Comments
L.O.I. (ORGANIC MATTER %)	14.3	% 15-20	okay
pH	5.7	5.8 minimum	low
CONDUCTIVITY (uS)	75	50	good
PHOSPHORUS (ppm)	15	30	low
POTASSIUM (ppm)	180	300	low
CALCIUM (ppm)	1380	3000	low
MAGNESIUM (ppm)	250	250	good
MANGANESE (ppm)	9	25	low
ZINC (ppm)	0.7	2.0	low
COPPER (ppm)	0.2	0.3	okay
BORON (ppm)	3.5	3.0	good

RECOMMENDATIONS:

Soil Acidity and Lime: 6 tonne /ha of lime to raise soil pH

Pre Planting Fertiliser: Apply (3-15-13-1) @ 1300 kg/ ha,

Which is the equivalent of : N 40: P 200: K 170: S 13 Kg/ ha

Trace Elements: Zinc Sulphate @ 3.5 kg / ha

Planting Fertiliser: Drill with 125 kg / ha of triple super
If White Root Rot present:
Drill with a mixture of 25% triple super and 75% of 50:50 lime and superphosphate treated with 1 L of Folicur at a rate of 125 kg / ha

12.11 Disease gradient analysis input code for Genstat®

```
EXPRESSION e1;VALUE=!e(Y=A*distance**B)
PRINT 'Power law model: Disease = A*distance^B'
MODEL prop_ve;FITTED=Y
RCYCLE PARAMETER=A,B;INITIAL=-1,1
FITNONLINEAR[CALCULATION=e1;PRINT=model,summary,esti;FPROB=y;CO
NSTANT=omit]
RGRAPH[GRAPHICS=high;TITLE='Prop+ve = A*Distance^B']distance
RCHECK[RMETHOD=pearson;GRAPHICS=high]residual;composite

EXPRESSION e2;VALUE=!e(Z=A*EXP(distance*(-1*B)))
PRINT 'Negative exponential model: Disease = A x exp(-B*distance)'
MODEL prop_ve;FITTED=Z
RCYCLE PARAMETER=A,B;INITIAL=1,1
FITNONLINEAR[CALCULATION=e2;FPROB=y;CONSTANT=omit;PRINT=mod
el,summary,esti]
RGRAPH [GRAPHICS=high;TITLE='Prop+ve = A exp(-B*Distance)'] distance
RCHECK [RMETHOD=pearson; GRAPHICS=high] residual; composite
```

12.12 Analysis of quest spore trap and weather data

Analysis of Quest quantitative spore trap data from Field trial 2m using Genstat®. The abbreviations used in the analysis of the Quest spore trap and weather data are explained in the following legend (Table 12.1). The numbers indicated in the abbreviations are the number of lag days between weather parameters and Quest spore trap data tested.

Table 12.1: Legend for the analysis of Quest spore trap and weather data

Abbreviation	Parameter
rfall	Rainfall
lfwet	Leaf wetness
Temp	Temperature
RH	Relative humidity
spores/m^3	Spores trapped per m^3 by Quest spore trap

*** Correlation matrix ***

spores/m^3	1.000				
rfall-5	0.281	1.000			
lfwet-5	0.100	0.393	1.000		
Temp-5	0.232	-0.079	-0.333	1.000	
RH-5	-0.220	0.117	0.475	-0.827	1.000
	spores/m^3	rfall-5	lfwet-5	Temp-5	RH-5

*** Correlation matrix ***

spores/m^3	1.000				
RH-4	-0.078	1.000			
Temp-4	0.178	-0.819	1.000		
lfwet-4	-0.096	0.509	-0.371	1.000	
rfall-4	-0.069	0.149	-0.026	0.312	1.000
	spores/m^3	RH-4	Temp-4	lfwet-4	rfall-4

*** Correlation matrix ***

spores/m^3	1.000				
RH-3	-0.025	1.000			
Temp-3	0.219	-0.812	1.000		
lfwet-3	0.133	0.507	-0.356	1.000	
rfall-3	0.405	0.147	-0.025	0.312	1.000
	spores/m^3	RH-3	Temp-3	lfwet-3	rfall-3

*** Correlation matrix ***

spores/m^3	1.000				
RH-2	-0.096	1.000			
Temp-2	0.125	-0.822	1.000		
lfwet-2	0.000	0.501	-0.381	1.000	
rfall-2	0.018	0.100	-0.020	0.280	1.000
	spores/m^3	RH-2	Temp-2	lfwet-2	rfall-2

*** Correlation matrix ***

spores/m^3	1.000				
RH-1	-0.083	1.000			
Temp-1	0.075	-0.829	1.000		
lfwet-1	-0.191	0.494	-0.416	1.000	
rfall-1	-0.025	0.006	0.001	0.195	1.000
	spores/m^3	RH-1	Temp-1	lfwet-1	rfall-1

Regression analysis, using parameters selected from the correlation analysis.

***** Regression Analysis *****

Response variate: spores/m^3

Fitted terms: Constant, RH-5, Temp-5, Temp-3, lfwet-1, lfwet-3, rfall-3, rfall-5

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	7	437353.	62479.	17.35	<.001
Residual	338	1217321.	3602.		
Total	345	1654674.	4796.		

Percentage variance accounted for 24.9

Standard error of observations is estimated to be 60.0

*** Estimates of parameters ***

	estimate	s.e.	t(338)	t pr.
Constant	-6.6	44.6	-0.15	0.883
RH-5	-0.299	0.329	-0.91	0.365
Temp-5	2.07	1.06	1.95	0.052
Temp-3	1.096	0.885	1.24	0.216
lfwet-1	0.095	0.849	0.11	0.911
lfwet-3	1.998	0.805	2.48	0.014
rfall-3	128.7	23.1	5.57	<.001
rfall-5	32.4	12.4	2.62	0.009

Dropping out some variables and re-running regression analysis.

Drop lfwet-1, RH-5, temp-3

Response variate: spores/m^3

Fitted terms: Constant, Temp-5, lfwet-3, rfall-3, rfall-5

*** Summary of analysis ***

	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	4	425395.	106349.	29.50	<.001
Residual	341	1229279.	3605.		
Total	345	1654674.	4796.		
Change	-3	11958.	3986.	1.106	0.347

Percentage variance accounted for 24.8

Standard error of observations is estimated to be 60.0

*** Estimates of parameters ***

	estimate	s.e.	t(341)	t pr.
Constant	-36.1	11.6	-3.11	0.002
Temp-5	3.550	0.571	6.21	<.001
lfwet-3	1.738	0.762	2.28	0.023
rfall-3	134.6	22.9	5.88	<.001
rfall-5	29.7	12.1	2.45	0.015

Spores/m^3 = -36.1 + 3.55 Temp-5 + 1.738 lfwet-3 + 134.6 rfall-3 + 29.7 rfall-5

(R^2 = 0.248)

12.13 Molecular methods

12.13.1 2% CTAB Buffer

for 200 ml	Final concentrations
8 ml (0.5M EDTA pH 8.0)	0.02 M
11.3 ml (1.77 M Tris pH 8.0)	0.1 M
16.36 g NaCl	1.4 M
180.7 ml MilliQ water	
4 g CTAB	2%

Activate 2% CTAB with 2.5% PVP, dissolve with heat and make fresh each time.

12.13.2 TE buffer (Tris/EDTA)

For 500 ml

0.6 g Tris in 400 ml	0.1 M
1.0 ml (0.5 M EDTA pH 8.0)	

Make up to 500 ml with MilliQ water, then autoclave

0.5 M EDTA pH 8.0

For 500 ml

To 300 ml of MilliQ water add:

93.05 g EDTA-$Na_2.2H_2O$

Mix well

Add NaOH until pH 8.0 (will not go into solution until about pH 7)

Add MilliQ water to 500 ml

Note: This solution stores better in the dark as it reduces the rate of precipitation. If precipitation occurs over time discard solution.

12.13.3 TAE buffer

Used in electrophoresis gels, and as running buffer.

50x TAE Stock (1 litre) (2.0 M tris, 1 M Acetate, 100 mM EDTA)

Tris base (mw 121.1)	242.0 g
Glacial acetic acid (17.4 M)	57.1 ml
EDTA (disodium, dihydrate) (mw 372.2)	37.2 g

Combine the Tris base and the EDTA first, then add distilled water to about 850 ml, dissolve reagents and add the acetic acid. Check and adjust the pH to 8.5 if necessary and add distilled water to 1000 ml.